IFW

FULBRIGHT & JAWORSKI L.L.P.
A REGISTERED LIMITED LIABILITY PARTNERSHIP
600 CONGRESS AVENUE, SUITE 2400
AUSTIN, TEXAS 78701-3271
WWW.FULBRIGHT.COM

GSHISHIMA@FULBRIGHT.COM
DIRECT DIAL: (512) 536-3081

TELEPHONE: (512) 474-5201
FACSIMILE: (512) 536-4598

December 21, 2004

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

December 21, 2004
Date

Gina N. Shishima

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Re: *SN 10/017,472 entitled "Methods of Treatment Involving Human MDA-7" by Chada et al.*
Our ref: INGN:097US

Commissioner:

Enclosed please find the following for filing in the above-referenced patent application:

1. Petition from Requirement for Restriction Pursuant to 37 C.F.R. §1.144; and

2. A return postcard to acknowledge receipt of these materials. Please date-stamp and mail this postcard.

It is believed that no fees are due. However, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed materials, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

Very truly yours,

Gina N. Shishima
Reg. No. 45,104

GNS/ld
Enclosures

25484831.1

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

December 21, 2004
Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

PETITION FROM REQUIREMENT FOR RESTRICTION PURSUANT TO 37 C.F.R. 1.144

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted pursuant to 37 C.F.R. § 1.144 in response to the Office Communications dated February 24, 2003, and June 3, 2003.

REMARKS

Applicants are petitioning the denial that claims 1 and 36 are proper linking claims.

A. Background

In a Restriction Requirement Dated February 24, 2003, the Examiner set forth four groups for restriction. Group I stated: "Claims 1-43 are drawn to a method for inhibiting

angiogenesis or endothelial cell differentiation comprising administering to a patient a nucleic acid expressing the human MDA-7 polypeptide." Exhibit A, page 2 (emphasis added). Group II stated: "Claims 1-6 and 13-41 are drawn to a method comprising administering to a patient a human MDA-7 polypeptide." Page 2 (emphasis added).

In the response to this Restriction filed on March 11, 2003, Applicants elected Group I and stated that "claims 1 and 36 were proper linking claims covering both Groups I and II." Exhibit B, page 2. Applicants also said, "If claim 1 or claim 36 is found to be allowable, the Group II claims must be rejoined and considered for allowance." *Id.*

In the Office Action Dated September 30, 2003, the Examiner stated:

> . . . Applicants are reminded that the restriction is not issued as linking claim type, because every invention recited in claims 1 and 36 are embraced by groups II and I. Each of the inventions requires a separate search status and consideration. The inventions are mutually exclusive and independent methods for *in vivo* gene and protein therapies. Therefore, it is maintained that these inventions are distinct due to their divergent subject matter.

Exhibit C, page 2. Moreover, in that Office Action, the Examiner indicated that claims 1, 13, 18-23, and 36-38 were objected to because they encompassed more than one invention as defined in the Restriction Requirement. The Action stated that claims should be amended to read upon only the elected invention. Exhibit C, page 3.

In the Response to the Office Action Dated September 30, 2003, Applicants urged reconsideration of the issue regarding claims 1 and 36 by traversing the objection to claims 1, 13, 18-23, and 36-38. Exhibit D, page 9. Applicants reiterated that claims 1 and 36 were proper linking claims. *Id.*

Now, Applicants are petitioning the restriction to the extent there has been a denial that claims 1 and 36 are proper linking claims and request the rejoinder of Group II if claim 1 or

claim 36 is found allowable. They further petition the objection to claims 1, 13, 18-23, and 36-38 as requiring amendment to reflect the election of Group I.

B. Claims 1 and 36 Are Proper Linking Claims

Claim 1 reads:

> A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

Claim 36 reads:

> A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid molecule expressing the human MDA-7 polypeptide.

First, Applicants note that administering a polypeptide and administering a nucleic acid expressing that polypeptide are not necessarily mutually exclusive. In fact, their relationship is similar to that of many species, meaning that one can be used instead of the other but they are not necessarily mutually exclusive.

Second, Applicants note that one of the most common linking claims is a "genus claim linking species claims." MPEP § 809.03. Claims 1 and 36 are genus claims linking the different species of administering an MDA-7 polypeptide and administering a nucleic acid molecule expressing the human MDA-7 polypeptide. Moreover, each of these species is clearly a way of generally providing MDA-7 polypeptide to the patient.

The linked claims "must be examined with any one of the linked inventions that may be elected." M.P.E.P. § 814. The MPEP specifically indicates:

> Where the requirement for restriction in an application is predicated upon the nonallowability of generic or other type of linking claims, applicant is entitled to

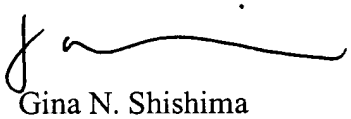
retain in the case claims to the nonelected invention or inventions. MPEP §809.04.

Therefore, it is inappropriate for the Examiner to request that any portion of the claim be amended merely based on the election of invention, as was set forth in the Response to Office Action filed on January 30, 2004. Exhibit D, page 9. In that Response, Applicants specifically indicated that withdrawal of claims was inappropriate and that they traversed the objection to claims 1, 13, 18-23, and 36-38.

Therefore, Applicants petition that claims 1 and 36 be recognized as linking claims and that Group II be re-joined if claim 1 or claim 36 is found allowable. Moreover, Applicants request clarification that Applicants need not amend the claims at this time to reflect the initial election of Group I.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097.

Respectfully submitted,



Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: December 21, 2004

EXHIBIT A

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590 02/24/2003

Gina N. Shishima
Fulbright & Jaworski L.L.P.
Suite 2400
600 Congress Avenue
Austin, TX 78701

EXAMINER	
LI, QIAN J	

ART UNIT	PAPER NUMBER
1632	

DATE MAILED: 02/24/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/017,472	CHADA ET AL.	
	Examiner	Art Unit	
	Q. Janice Li	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *12/7/01* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-67* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-67* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO-1449) Paper No(s) _____ .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S. C. 121:

I. Claims 1-43 are drawn to a method for inhibiting angiogenesis or endothelial cell differentiation comprising administering to a patient a nucleic acid expressing the human MDA-7 polypeptide. Classified in Class 514, subclass 44.

II. Claims 1-6, and 13-41 are drawn to a method comprising administering to a patient a human MDA-7 polypeptide. Classified in Class 514, subclass 2.

III. Claims 44-67 are drawn to a method for promoting an immune response comprising administering to a patient a nucleic acid expressing the human MDA-7 polypeptide and an antigen. Classified in Class 514, subclass 44, and class 424, subclass 184.1.

IV. Claims 44-51, and 56-67 are drawn to a method for promoting an immune response comprising administering to a patient a human MDA-7 polypeptide and an antigen. Classified in Class 514, subclass 2, and class 424, subclass 184.1.

2. The inventions are distinct, each from the other because of the following reasons.

Inventions II-IV and I are independent and distinct inventions. Inventions are distinct if it can be shown that they are not disclosed as capable of use together and they have different modes of operation, different functions, or different effects (MPEP § 806.04, MPEP § 808.01). In the instant case, each of the groups I-IV are drawn to a different method of inhibiting angiogenesis, endothelial cell differentiation, and promoting immune response. Each of the

groups differs in the starting material used in the process and mode of operation as well as biological effects. For example, a nucleic acid has distinct biodistribution and pharmacokinetics compared to a polypeptide, the antigens used in groups III and IV are not used in groups I and II. Therefore, the different groups of invention require distinct technical considerations and search criteria.

The differences of the Inventions I-IV are further underscored by their divergent classification and independent search criteria.

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art because of their recognized divergent subject matter and different search criteria, it would impose an undue burden to the Office if all the groups are examined together, thus, restriction for examination purposes as indicated is proper.

3. This application contains claims directed to the following patentably distinct species of the claimed invention: i.e. different types of diseases that need to be treated, different viral vectors used in the methods, different type of antigens for administration, and different fragments of MDA-7 polypeptides. Upon election of an invention for examination in this application, further election of a species is necessary, i.e. select a particular disease, a particular MDA-7 fragment, and if applicable, select a particular type of antigen and a particular type of vector for examination

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-67 are generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

4. Applicant is advised that the reply to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed (37 CFR 1.143).

Applicant is advised that where a single claim encompasses more than one invention as defined above, upon election of an invention for examination, said claim will only be examined to the extent that it reads upon the elected invention.

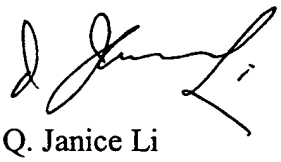
5. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a petition under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Q. Janice Li whose telephone number is 703-308-7942. The examiner can normally be reached on 8:30 am - 5 p.m., Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah J. Reynolds can be reached on 703-305-4051. The fax numbers for the organization where this application or proceeding is assigned are 703-872-9306 for regular communications and 703-872-9307 for After Final communications.

Any inquiry of formal matters can be directed to the patent analyst, Dianiece Jacobs, whose telephone number is (703) 305-3388.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-1235. The faxing of such papers must conform to the notice published in the Official Gazette 1096 OG 30 (November 15, 1989).



Q. Janice Li
Examiner
Art Unit 1632

QJL
February 21, 2003

EXHIBIT B

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date below:

March 11, 2003
Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

RESPONSE TO RESTRICTION REQUIREMENT DATED FEBRUARY 24, 2003

Commissioner for Patents
Washington, D.C. 20231

Commissioner:

This paper is submitted in response to the Restriction Requirement dated February 24, 2003 for which the date for response is March 24, 2003.

It is believed that no fee is due; however, should any fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to this document, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

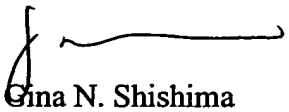
In response to the restriction requirement that the Examiner imposed, Applicants elect, without traverse, to prosecute claims 1-43, *i.e.*, the Group I claims.

Claims 1 and 36 are proper linking claims covering both Groups I and II. If claim 1 or claim 36 is found to be allowable, the Group II claims must be rejoined and considered for allowance.

Consistent with this restriction, Applicants will cancel claims 44-67.

The Examiner is invited to contact the undersigned attorney at (512) 536-3081 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: March 11, 2003

EXHIBIT C

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590 09/30/2003

Gina N. Shishima
Fulbright & Jaworski L.L.P.
Suite 2400
600 Congress Avenue
Austin, TX 78701

EXAMINER	
LI, QIAN J	

ART UNIT	PAPER NUMBER
1632	13

DATE MAILED: 09/30/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
10/017,472	12/07/2001	Sunil Chada	INGN:097US	5209

7590 09/30/2003

Gina N. Shishima
Fulbright & Jaworski L.L.P.
Suite 2400
600 Congress Avenue
Austin, TX 78701

EXAMINER	
LI, QIAN J	

ART UNIT	PAPER NUMBER
1632	13

DATE MAILED: 09/30/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

Office Action Summary	Application No.	Applicant(s)	
	10/017,472	CHADA ET AL.	
	Examiner	Art Unit	
	Q. Janice Li	1632	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *07 July 2003* .

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-25,32-43 and 68-74* is/are pending in the application.

4a) Of the above claim(s) *5,6 and 68-74* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *1-4,7-25,32-43* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☒ The drawing(s) filed on *07 December 2002* is/are: a)☒ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

11)☐ The proposed drawing correction filed on _____ is: a)☐ approved b)☐ disapproved by the Examiner.

If approved, corrected drawings are required in reply to this Office action.

12)☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

13)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____ .

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

14)☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).

a) ☐ The translation of the foreign language provisional application has been received.

15)☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) *11* .
4) ☐ Interview Summary (PTO-413) Paper No(s). _____ .
5) ☐ Notice of Informal Patent Application (PTO-152)
6) ☐ Other: .

DETAILED ACTION

Election/Restrictions

Applicant's election without traverse of Group I, drawn to a method of using a nucleic acid encoding and expressing MDA-7, in Paper No. 9, and supplemental election of species, drawn to a method of treating angiogenesis-dependent cancer using an adenoviral vector expressing fragment 182-206 of SEQ ID No: 2, in Paper No. 12 is acknowledged. In paper #9, applicants indicated that claims 1 and 36 are linking claims of group II and I, upon allowance of group I, claims of group II should be rejoined. In response, Applicants are reminded that the restriction is not issued as linking claim type, because every invention recited in claims 1 and 36 are embraced by groups II and I. Each of the Inventions requires a separate search status and consideration. The inventions are mutually exclusive and independent methods for *in vivo* gene and protein therapies. Therefore, it is maintained that these inventions are distinct due to their divergent subject matter. Further search of these inventions is not co-extensive, as indicated by the separate classifications. The requirement is still deemed proper and is therefore made **FINAL**.

Please note that after a final requirement for restriction, the Applicants, in addition to making any response due on the remainder of the action, may petition the Commissioner to review the requirement. Petition may be deferred until after final action on or allowance of claims to the invention elected, but must be filed not later than

appeal. A petition will not be considered if reconsideration of the requirement was not requested. (See § 1.181.).

Claims 26-31 and 44-67 have been cancelled, claim 6 has been amended, and claims 68-74 are newly submitted. Claims 1-25, 32-43, and 68-74 are pending, however, claims 5, 6, and 68-74 are withdrawn from further consideration by the Examiner, pursuant to 37 CFR 1.142(b), as being drawn to non-elected inventions, there being no allowable generic or linking claim. Claims 1-4, 7-25, 32-43 are under current examination.

Claim Objections

Claims 1, 13, 18-23, 36-38 are objected to because of the following informalities: claims encompass more than one invention as defined in Paper #8, upon election of an invention for examination, said claims should be amended so that they only read upon the elected invention.

Claim 1 is objected to because of the claim recitation, "MDA-7". The abbreviation should be spelled out the first time it appears in the claims.

Claims 16 and 17 are objected to under 37 CFR 1.75(c), as being of improper dependent form for failing to further limit the subject matter of a previous claim. Specifically, claims 16 and 17 are directed to injections performed distally to a disease site, yet depends from a claim directed to local injection (claim 13). Applicant is required to amend the claim(s) to place the claim(s) in proper dependent form, or rewrite the claim(s) in independent form.

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claims 1-4, 7-25, 32-43 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for *intratumoral injection* of a nucleic acid expressing *full length* MDA-7 polypeptide for treating angiogenesis-dependent cancer, wherein the MDA polypeptide *lacks* a secretory signal, does not reasonably provide enablement for distal or systemic administration of an adenoviral vector expressing *fragments* of MDA-7 polypeptide for treating angiogenesis-dependent tumor, and wherein the MDA-7 polypeptide comprising a secretory signal. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to practice the invention commensurate in scope with these claims.

The factors to be considered when determining whether the disclosure satisfies the enablement requirements and whether undue experimentation would be required to make and use the claimed invention are summarized in *In re Wands*, (858 F2d 731, 737, 8 USPQ 2d 1400, 1404, (Fed Cir.1988)). These factors include but are not limited to the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability of the art, the breadth of the claims, and amount of direction provided. The factors most relevant to this rejection are the scope of the claims relative to the state of the art and the levels of the skilled in the art, and whether sufficient

amount of direction or guidance are provided in the specification to enable one of skill in the art to practice the claimed invention.

Given the broadest reasonable interpretation, the claims encompass treating cancer with fragments of MDA-7. The specification contemplates that truncated MDA-7 is part of the invention, which encompass fragments ranging from 10 to 206 contiguous amino acids of SEQ ID No: 2 (Specification, page 13, lines 16-20, for example). However, neither the specification, nor art of record, teaches a consensus region that is critical for the function of MDA-7 or the structural correlation of the polypeptide with its function for inhibiting the growth of tumor cells, and accordingly the specification does not provide a reasonable guide for those seeking to practice the invention. This is because the art of protein chemistry is one of the most unpredictable areas of biotechnology. Although the polynucleotide-coding region determines amino acid sequence of the protein, it is the conformation of three-dimensional structures that forms active site, allows the protein to function, and carry out the messages of the genome. *Bowie et al* (Science 1990 Mar; 247:1306-10) teach certain position in the sequence are critical to the three dimensional structure/function relationship and these regions can tolerate only conservative substitutions or none at all (page 1306, column 2). *Skolnick et al* (TIBTECH 2000 Jan;18:34-9) teach, "SEQUENCE-BASED METHODS FOR FUNCTION PREDICTION ARE INADEQUATE BECAUSE OF THE MULTIFUNCTIONAL NATURE OF PROTEINS. HOWEVER, JUST KNOWING THE STRUCTURE OF THE PROTEIN IS ALSO INSUFFICIENT FOR PREDICTION OF MULTIPLE FUNCTIONAL SITES" (abstract). They further teach, "KNOWING A PROTEIN'S THREE-DIMENSIONAL STRUCTURE IS INSUFFICIENT TO DETERMINE ITS FUNCTION" (box 1, page 35). Thus,

one cannot predictably extrapolate the teachings of the specification to the scope of the claims because the skilled artisan cannot envision the detailed structure of fragments of SEQ ID No: 2 encompassed by these claims with the function of the fragments. Moreover, it is unclear exactly what modifications and variations can be tolerated in this protein and still allows proper tumor-inhibiting function. Determination of the effects of particular modifications and fragmentations are not predictable until they are actually made and used, hence resulting in a trial and error situation. *Rudinger* (Peptide Hormones 1976; June;pages 1-7) teaches the relationship of sequence components and the peptide hormone function "THE SIGNIFICANCE OF PARTICULAR AMINO ACIDS AND SEQUENCES FOR DIFFERENT ASPECTS OF BIOLOGICAL ACTIVITY CANNOT BE PREDICTED *A PRIORI* BUT MUST BE DETERMINED FROM CASE TO CASE BY PAINSTAKING EXPERIMENTAL STUDY." (last paragraph of text on page 6). The specification fails to provide sufficient teaching for the fragments of MDA-7, it would have required undue experimentation for the skilled artisan intending to practice the instant invention.

With respect to the secretory signal, *Su et al* (PNAS 1998;9514400-5, IDS/C65) teach that the tumor-suppressing effect of mda-7 is associated with chromatin remodeling via its nucleus translocation from the cytosol, and facilitating the migration of mda-7 into the nucleus would enhance the selective growth inhibition of malignant but not normal cells (§ Discussion, page 14404). In view of such teaching, addition of a secretory signal on MDA-7 polypeptide, would prohibit the nucleus translocation, thus may abolish the anti-tumor effect of mda-7. In view of such, the invention does not

appear to be enabled in the absence of clarification of the contradictory evidence found in the cited references.

Claims also contemplate administering a (*any*) nucleic acid, naked or in any type of vector, particularly adenoviral vector encoding mda-7 through regional and systemic delivery from a site *distal* from the site of the disease. However, the specification fails to teach how the nucleic acid could reach the target site in a sufficient amount so that a therapeutic effect of tumor killing would achieve. While progress has been made in recent years for gene transfer *in vivo*, vector targeting to desired cells *in vivo* continues to be unpredictable and inefficient as supported by numerous teachings available in the art. For example, *Deonarain* (1998, Expert Opin. Ther. Pat., Vol. 8, pages 53-69) indicate that one of the biggest problems hampering successful gene therapy is the "ABILITY TO TARGET A GENE TO A SIGNIFICANT POPULATION OF CELLS AND EXPRESS IT AT ADEQUATE LEVELS FOR A LONG ENOUGH PERIOD OF TIME" (page 53, first paragraph). Deonarain reference gives high hope to targeted gene delivery, but the discussed strategies are still under investigation, and at the time, they were much less efficient than viral gene delivery (Conclusion).

The claims are drawn to using any naked polynucleotides and vectors. However, whether the recited vectors are suitable for the purpose of the instant invention are unclear. For example, adenoviral vectors are known for their tissue tropism of respiratory epithelial cells, which would be a critical limitation for targeting any angiogenesis-dependent cancer. *Miller et al* (1995, FASEB J., Vol. 9, pages 190-199), acknowledge various vector system available in the art, then teach, "NO SINGLE DELIVERY

SYSTEM IS LIKELY TO BE UNIVERSALLY APPROPRIATE, FOR INSTANCE, THE REQUIREMENTS OF GENE THERAPY FOR CYSTIC FIBROSIS ARE GREATLY DIFFERENT FROM THOSE OF CANCER" (1st paragraph, page 190). "ONCE AGAIN, TARGETING AT THE LEVEL OF THE VECTOR HAS NOT YET BEEN PARTICULARLY WELL DEVELOPED; HENCE, LIPOSOME OR VIRAL-MEDIATED DELIVERY OF THE CFTR GENE TO AIRWAY EPITHELIAL CELLS OF CF PATIENTS HAS RELIED LARGELY ON THE LOCALIZED DELIVERY OF THE VECTORS DIRECTLY TO THE AFFECTED TISSUES" (1st paragraph, page 198) *Makrides et al* (Protein Exp Pur 1999;17:183-202) teach "THE CHOICE OF AN EXPRESSION SYSTEM FOR PRODUCTION OF RECOMBINANT PROTEINS DEPENDS ON MANY FACTORS, INCLUDING CELL GROWTH CHARACTERISTICS, EXPRESSION LEVELS, INTRACELLULAR AND EXTRACELLULAR EXPRESSION, POSTTRANSLATIONAL MODIFICATIONS AND BIOLOGICAL ACTIVITY OF THE PROTEIN OF INTEREST, AS WELL AS REGULATORY ISSUES AND ECONOMIC CONSIDERATIONS IN THE PRODUCTION OF THERAPEUTIC PROTEINS." *Boucher et al* (J Clin Invest 1999 Feb; 103:441-5) review that host cell resistance to foreign gene is another difficulty for successful in vivo gene transfer. "DESPITE AN IMPRESSIVE AMOUNT OF RESEARCH IN THIS AREA, THERE IS LITTLE EVIDENCE TO SUGGEST THAT AN EFFECTIVE GENE-TRANSFER APPROACH FOR THE TREATMENT OF CF LUNG DISEASE IS IMMINENT. THE INABILITY TO PRODUCE SUCH A THERAPY REFLECTS IN PART THE LEARNING CURVE WITH RESPECT TO VECTOR TECHNOLOGY AND THE FAILURE TO APPRECIATE THE CAPACITY OF THE AIRWAY EPITHELIAL CELLS TO DEFEND THEMSELVES AGAINST THE PENETRATION BY MOIETIES, INCLUDING GENE-THERAPY VECTORS, FROM THE OUTSIDE WORLD." The specification fails to teach how to overcome the aforementioned difficulties in the art. It would have required undue experimentation for the skilled artisan intending to practice the instant invention.

Thus, it is evident that at the time of the invention, the gene therapy practitioner, while acknowledging the significant potential of gene therapy for cancer, still recognized

that such therapy was neither routine nor accepted, and awaited significant development and guidance for its practice. Therefore, it is incumbent upon applicants to provide sufficient and enabling teachings within the specification for such therapeutic regimen. Although the instant specification provides a brief review of a potential therapeutic use of the claimed method and data from ex vivo and animal studies, it is not enabled for its full scope because the specification does not disclose the structural-function relationship of MDA-7 fragments, whether the nucleic acids encompassed by the claims would function properly in vivo by any means of delivery.

Accordingly, in view of the quantity of experimentation necessary to determine the parameters for achieving *in vivo* gene expression in selected cells at therapeutic levels, in particular with any fragment of MDA-7 and any type of nucleic acids, the lack of direction or guidance provided by the specification as well as the absence of working examples with regard to targeted *in vivo* gene therapy with fragments of MDA-7 delivered by regional and systemic routes, and the breadth of the claims directed to the use of numerous fragments, it would have required undue experimentation for one skilled in the art to make and/or use the claimed invention.

The following is a quotation of the second paragraph of 35 U.S.C. 112:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claim 9 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 9 is vague and indefinite because of the unit information is incomplete. The recited "pfu" could be the viral stock solution of "pfu per mL" or the infected cell concentration, "pfu per cell", it is unclear which one the applicants intend to claim, and thus the metes and bounds of the claim are uncertain.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, 43 are rejected under 35 U.S.C. 102(e) as being anticipated by *Fisher* (US 6,355,622).

Fisher teaches a method of inhibiting angiogenesis dependent cancer in a subject suffering from cancer comprising intratumoral administering to nude mice bearing human cervical carcinoma cells replication deficient adenoviral vector encoding mda-7 gene (AA 1-206 of SEQ ID No. 2) three times a week for 4 weeks, the well-established tumors were growth inhibited in the treated mice compared to the control group (column 14, lines 35-67), wherein the expression of mda-7 was driven by a CMV promoter (column 13, line 56). *Fisher* also teaches that the nucleic acid could be embedded in liposomes and introduced into the cell (column 3, line 67, lipid

composition). *Fisher* teaches that ectopic expression of mda-7 inhibits the growth of tumor cells and may provide therapeutic benefit for the treatment of human cancer (column 14, lines 62-65). Therefore, *Fisher* anticipates the instant claims.

Claims 1-4, 7-25, 35-43 are provisionally rejected under 35 U.S.C. 102(e) as being anticipated by copending Application No. 09/615,154 which has a common inventor with the instant application. Based upon the earlier effective U.S. filing date of the copending application, it would constitute prior art under 35 U.S.C. 102(e), if published under 35 U.S.C. 122(b) or patented. This provisional rejection under 35 U.S.C. 102(e) is based upon a presumption of future publication or patenting of the copending application.

Claims of instant application and the cited application are each drawn to a method of treating a tumor patient comprising administering a viral vector expressing a mda-7 polypeptide or fragment 182-206 of SEQ ID No:2 combined with conventional chemotherapy, surgery, and radiation therapy. Considerable overlap in the scope of the claims is present. Therefore, the inventions as claimed are co-extensive.

This provisional rejection under 35 U.S.C. 102(e) might be overcome either by a showing under 37 CFR 1.132 that any invention disclosed but not claimed in the copending application was derived from the inventor of this application and is thus not the invention "by another," or by an appropriate showing under 37 CFR 1.131. This rejection may not be overcome by the filing of a terminal disclaimer. See *In re Bartfeld*, 925 F.2d 1450, 17 USPQ2d 1885 (Fed. Cir. 1991).

Claims 1-4, 7-25, 35-43 are rejected under 35 U.S.C. 102(f) because the applicant did not invent the claimed subject matter. U.S. patent application 09/615,154 has a different inventive entity, yet the disclosure anticipates the instantly claimed invention. It is unclear as to who is the real inventor. Appropriate clarification is required.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

Claims 1, 7-9, 20-23, 36-41 are rejected under 35 U.S.C. 103(a) as being unpatentable over *Roth et al* (US 6,069,134), in view of *Fisher* (US 6,355,622).

Roth et al teach a method comprising administering DNA damaging agent combined with adenoviral vector expressing a tumor suppressor (particularly p53, abstract), together with conventional chemotherapy and surgery for the treatment of cancer (column 3, lines 20-48). *Roth et al* teach that the DNA damaging agents include gamma-irradiation, x-rays and UV-irradiation, for example; and the chemotherapeutic agents include 5-fluorouracil (column 4, lines 57-67). *Roth et al* also teach that the adenoviral stock was administered at a m.o.i. of 10^8 pfu/ml (column 12, line 1). *Roth et al* do not teach that the tumor suppressor is MDA-7.

Fisher teaches that using adenovirus encoding MDA-7 as the tumor suppressor for treatment of cancer, and administering the vector to tumor cells *in vitro* at moi of 10^2pfu/cell, but does not specify the dosage for *in vivo* administration (column 14, line 22). *Fisher* teaches that ectopic expression of mda-7 inhibit the growth of tumor cells and may provide therapeutic benefit for the treatment of human cancer in general, but did not discuss the details of such therapy (column 14, lines 62-65).

Claims 20-23 and 37-41 are limitations for the timing of the combination therapy, neither Roth et al nor Fisher discuss the details. However, given the levels of the ordinary skilled in the art, these limitations would fall within the bounds of the optimization for a proper therapeutic regimen.

Accordingly, it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the methods taught by *Roth et al* by simply substituting the p53 with mda-7 as taught by *Fisher et al* and administering the mda-7 either prior or after the conventional therapy at a dosage sufficient for tumor cell killing with a reasonable expectation of success. The ordinary skilled artisan would have been motivated to modify the claimed invention because the combined therapy would maximize the tumor-treating effect by any individual therapy alone. Thus, the claimed invention as a whole was *prima facie* obvious in the absence of evidence to the contrary.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the

unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970);and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claims 1-4, 7-25, 32, and 35-43 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 91-116,125-154, 159-174 of copending U.S. Patent Application No. 09/615,154.

Although the conflicting claims are not identical, they are not patentably distinct from each other because the present application and the claims of the cited patent are each drawn to a method for treating a tumor patient comprising administering a viral vector expressing a mda-7 polypeptide combined with conventional chemotherapy, surgery, and radiation therapy. Considerable overlap in the scope of the claims is also present.

Accordingly, the claimed processes in the copending and the present application are obvious variants. Therefore, the inventions as claimed are co-extensive.

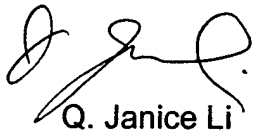
No claim is allowed.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Q. Janice Li whose telephone number is 703-308-7942. The examiner can normally be reached on 8:30 am - 5 p.m., Monday through Friday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Deborah J. Reynolds can be reached on 703-305-4051. The fax numbers for the organization where this application or proceeding is assigned are 703-872-9306 for regular communications and 703-872-9307 for After Final communications.

Any inquiry of formal matters can be directed to the patent analyst, Dianiece Jacobs, whose telephone number is (703) 305-3388.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-1235. The faxing of such papers must conform to the notice published in the Official Gazette 1096 OG 30 (November 15, 1989).



Q. Janice Li
Patent Examiner
Art Unit 1632

QL
September 22, 2003

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant	Applicant Sunil Chada *et al.*	
INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
	A1	4,682,195	7-21-87	Yilmaz	357	23.4	9-30-85
	A2	4,683,202	7-28-87	Mullis	435	91	10-25-85
	A3	4,797,368	1-10-89	Carter *et al.*	435	320	3-15-85
	A4	5,139,941	8-18-92	Muzyczka *et al.*	435	172.3	10-25-91
	A5	5,399,363	3-21-95	Liversidge *et al.*	424	490	7-1-92
	A6	5,466,468	11-14-95	Schneider *et al.*	424	450	10-28-94
	A7	5,543,158	8-6-96	Gref *et al.*	424	501	7-23-93
	A8	5,633,016	5-27-97	Johnson	424	649	5-1-95
	A9	5,641,515	6-24-97	Ramtoola	424	189	6-7-95
	A10	5,645,897	7-8-97	Andra	427	526	1-18-95
	A11	5,705,629	1-6-98	Bhongle	536	25.34	10-20-95
	A12	5,739,169	4-14-98	Ocain *et al.*	514	658	5-31-96
	A13	5,798,339	8-25-98	Brandes	514	34	6-28-93
	A14	5,801,005	9-1-98	Cheever *et al.*	435	7.24	3-31-95
	A15	5,824,311	10-20-98	Greene *et al.*	424	138.1	11-30-94
	A16	5,824,348	10-20-98	Fujiu *et al.*	425	120	1-16-97
	A17	5,830,880	11-3-98	Sedlacek *et al.*	514	44	4-18-97
	A18	5,846,225	12-8-98	Rosengart *et al.*	604	115	2-19-97
	A19	5,846,233	12-8-98	Lilley *et al.*	604	414	1-9-97
	A20	5,846,945	12-8-98	McCormick	514	44	6-7-95

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
	B1	266032	5-4-88	Europe			
	B2	WO 00/05356	2-3-00	PCT			
	B3	WO 00/26368	5-11-00	PCT			
	B4	WO 95/11986	5-4-95	PCT			
	B5	WO 98/07408	2-26-98	PCT			
	B6	WO 98/28425	7-2-98	PCT			

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C1	Anderson, "Human gene therapy," *Nature*, 392:25-30, 1998.
	C2	Austin-Ward and Villaseca, "Gene therapy and its applications," *Rev. Med. Chil.*, 126:838-845, 1998. Article in Spanish, Abstract included.
	C3	Blumberg *et al.*, "Inyrtlrukin 20: discovery, receptor identification, and role in epidermal function," *Cell* 104:9-19, 2001.
	C4	Bonavida *et al.*, "Selectivity of TRAIL-mediated apoptosis of cancer cells and synergy with drugs: The trail to non-toxic cancer therapeutics," *Int J Oncol*, 15:793-802, 1999.
	C5	Bukowski *et al.*, "Signal transducton abnormalities in T lymphyocytes from patients with advanced renal carcinoma: Clinical relevance and effects of cytokine therapy," *Clin. Cancer Res.*, 4(10):2337-2347, 1998.
	C6	Chinnaiyan *et al.*, "Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy," *Proc Nat'l Acad Sci USA*. 97:1754-1759, 2000.
	C7	Christodoulides *et al.*, "Immunization with recombinant class 1 outer-membrane protein from n*eisseria meningitidis*: influence of liposomes and adjuvants on antibody avidity, recognition of native protein and the induction of a bactericidal immune response against meningococci," *Microbiology*, 144(Pt 11):3027-3037, 1998.
	C8	Crystal, "Transfer of genes to humans: early lessons and obstacles," *Science*, 270:404-409, 1995.

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's Information Disclosure Statement (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C9	Davidson *et al.*, "Intralesional cytokine therapy in cancer: A pilot study of GM-CSF infusion in mesothelioma," *J. Immunother.*, 21:389-398, 1998.
	C10	De Waal Malefyt *et al.*, "Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes," *J. Exp. Med.* 174:1209-1220, 1991.
	C11	Dragovich *et al.*, "Signal transduction pathways that regulate cell survival and cell death," *Oncogene,* 17:3207-3213, 1998
	C12	Dumoutier *et al.*, "Human interleukin-10-related T cell-derived inducible factor: molecular cloning and functional characterization as an hepatocyte-stimulating factor," *Proc. Natl. Acad. Sci. USA.* 97:10144-10149, 2000.
	C13	Ekmekcioglu *et al.*, "Differential increase of Fas ligand expression on metastatic and thin or thick primary melanoma cells compared with interleukin-10," *Melanoma Research* 9:261-272, 1999.
	C14	Ekmekcioglu *et al.*, "Down-regulated melanoma differentiation associated gene (MDA-7) expression in human melanomas," *Intl. J. Cancer.*, 94:54-59, 2001
	C15	el-Kareh and Secomb, "Theoretical models for drug delivery to solid tumors," *Crit. Rev. Biomed. Eng.*, 25:503-571, 1997.
	C16	Erlandsson, "Molecular genetics of renal cell carcinoma," *Cancer Genet. Cytogenet,* 104:1-18, 1998.
	C17	Fulci *et al.*, "p53 and brain tumors: from gene mutations to gene therapy," *Brain Pathol.*, 8(4):599-613, 1998.
	C18	Gallagher *et al.*, "Cloning, expression and initial characterisation of interleukin-19 (IL-19), a novel homologue of human interleukin-10 (IL-10)," *Genes Immun.* 1:442-450, 2000.
	C19	GenBank Accession Number XM_001405
	C20	Gertig and Hunter, "Genes and environment in the etiology of colorectal cancer," *Semin. Cancer Biol.*, 8(4):285-298, 1997.
	C21	Gliniak and Le, "Tumor necrosis factor-related apoptosis-inducing ligand's antitumor activity *in* vivo is enhanced by the chemotherapeutic agent CPT-11," *Cancer Res.* 59:6153-6158, 1999.

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's Information Disclosure Statement (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C22	Han *et al.*, "The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-unducible and death-promoting Bax protein," *Genes Dev.*, 10(4):461-477, 1996.
	C23	Hanibuchi *et al.*, "Therapeutic efficacy of mouse-human chimeric anti-ganglioside gm2 monoclonal antibody against multiple organ micrometastases of human lung cancer in NK cell-depleted scid mice," *Int. J. Cancer*, 78:480-485, 1998.
	C24	Hartmann *et al.*, "High frequency of p53 gene mutations in primary breast cancers in Japanese women, a low-incidence population," *Br. J. Cancer*, 73(8):896-901, 1996.
	C25	Hartmann *et al.*, "Overexpression and mutations of p53 in metastatic malignant melanomas," *Int. J. Cancer*, 67(3):313-317, 1996.
	C26	Hellstrand *et al.*, "Histamine and cytokine therapy," *Acta. Oncol.*, 37:347-353, 1998.
	C27	Ho *et al.*, "Internal radiation therapy for patients with primary or metastatic hepatic cancer," *Cancer*, 83:1894-1907, 1998.
	C28	Howard *et al.*, "Biological properties of interleukin 10," *J. Clin. Immunol.* 12:239-247, 1992.
	C29	Hui and Hashimoto, "Pathways for potentiation of immunogenicity during adjuvant-assisted immunizations with *plasmodium falciparum* major merozoite surface protein 1," *Infect. Immun.*, 66:5329-3536, 1998.
	C30	Jiang and Fisher, "Use of a sensitive and efficient subtraction hybridization protocol for the identification of genes differentially regulated during the induction of differentiation in human melanoma cells," *Mol. Cel. Differ.*, 1(3): 285-299, 1993.
	C31	Jiang *et al.*, "Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression," *Oncogene*, 11:2477-2486, 1995.
	C32	Jiang *et al.*, "The melanoma differentiation associated gene *mda*-7 suppresses cancer cell growth," *Proc. Natl Acad. Sci. USA*, 93:9160-9165, 1996.
	C33	Johnson and Hamdy, "Apoptosis regulating genes in prostate cancer," *Oncol. Rep.*, 5:553-557, 1998.
	C34	Keane *et al.*, "Chemotherapy augments TRAIL-induced apoptosis in breast cell lines," *Cancer Res.* 59:734-741, 1999.

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/016,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C35	Knappe *et al.*, "Induction of a novel cellular homolog of interleukin-10, AK155, by transformation of T lymphocytes with herpesvirus saimiri," *J. Virol.*, 74: 3881-3887, 2000.
	C36	Kolmel, "Cytology of neoplastic meningosis," *J. Neurooncol.*, 38:121-125, 1998.
	C37	Kotenko *et al.*, "Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10)," *Proc. Natl. Acad. Sci. USA*, 97:1695-1700, 2000.
	C38	Levine *et al.*, "The p53 tumor suppressor gene," *Nature*, 351:453-456, 1991.
	C39	Liebermann *et al.*, "AP-1 (Fos/Jun) transcription factors in hematopoietic differentiation and apoptosis," *Int. J. Oncol.*, 12:685-700, 1998.
	C40	Magi-Galluzzi *et al.*, "Proliferation, apoptosis and cell cycle regulation in prostatic carcinogenesis," *Anal. Quant. Cytol. Histol.*, 20:343-350, 1998.
	C41	Mangray and King, "Molecular pathobiology of pancreatic adenocarcinoma," *Front Biosci.*, 3:D1148-1160, 1998.
	C42	Marsters *et al.*, "Control of apoptosis signaling by Apo2 ligand," *Recent Prog Horm Res* 54:225-234, 1999.
	C43	Mayer, "Future developments in the selectivity of anticancer agents: drug delivery and molecular target strategies," *Cancer Metastasis Rev.*, 17:211-8, 1998.
	C44	Mhashilkar *et al.*, "Melanoma differentiation associated gene-7 (mda-7): a novel anti-tumor gene for cancer gene therapy," *Mol. Medicine.* 7:271-282, 2001.
	C45	Miyashita and Reed, "Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene," Cell 80:293-299, 1995.
	C46	Moore *et al.*, "Homology of cytokine synthesis inhibitory factor (IL-10) to the epstein-barr virus gene BCRFI," *Science*, 248:1230-1234, 1990.
	C47	Mougin *et al.*, "Biologie des infections à papillomavirus II. Leur rôle dans la carcinogenese du col utérin," *Ann. Biol. Clin.* (Paris), 56:21-28, 1998, Abstract attached.
	C48	Mountain, "Gene threapy: the first decade," *TIBTECH*, 18:119-127, 2000.
	C49	Mumby and Walter, "Protein phosphatases and DNA tumor viruses: transformation through the backdoor?," *Cell Regul.*, 2:589-598, 1991.

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C50	Natoli *et al.*, "Apoptotic, non-apoptotic, anti-apoptotic pathways of tumor necrosis, factor signalling," *Biochem. Pharmacol.*, 56(8):915-920, 1998.
	C51	Ochi *et al.*, "A case of small pancreatic cancer diagnosed by serial follow-up studies promptly by a positive K-ras point mutation in pure pancreatic juice," *Am. J. Gastroenterol.*, 93:1366-1368, 1998.
	C52	Ohara, "Radiotherapy: a significant treatment option in m," *Gan. To. Kagaku. Ryoho.*, 25:823-828, 1998.
	C53	Pietras *et al.*, "Remission of human breast cancer xenografts on therapy with humanized monoclonal antibody to HER-2 receptor and DNA reactive drugs," *Oncogene*, 17:2235-49, 1998.
	C54	Qin *et al.*, "Interferon-β gene therapy inhibits tumor formation and causes regression of established tumors in immune-deficient mice," *Proc. Nat'l Acad. Sci. USA*, 95(24):1411-1416, 1998.
	C55	Reed *et al.*, "Structure-function analysis of Bcl-2 family proteins," *Adv. Exp. Med. Biol.*, 406:99-112, 1996.
	C56	Reed, "Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies," *Semin Hematol.*, 34(4 Suppl. 5):9-19, 1997.
	C57	Robbins and Ghivizzani, "Viral vectors for gene therapy," *Pharmacol. Ther.*, 80:35-47, 1998.
	C58	Rosse *et al.*, "Bcl-2 prolongs cell survival after bax-induced release of cytochrome c," *Nature* 391:496-499, 1998.
	C59	Schaefer *et al.*, "Cutting Edge: FISP (IL-4-induced secreted protein), a novel cytokine-like molecule secreted by Th2 cells," *J. Immunol.*, 166:5859-5863, 2001.
	C60	Sedlak *et al.*, "Multiple Bcl-2 family members demonstrate selective dimerizations with *bax*," *Proc. Nat'l. Acad. Sci. USA*, 92:7834-7838, 1995.
	C61	Shimizu *et al.*, "Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel vdac," *Nature*, 399:483-487, 1999.
	C62	Solyanik *et al.*, ""Different growth patterns of a cancer cell population as a function of its starting growth characteristics: analysis by mathematical modeling," *Cell Prolif.*, 28:263-278, 1995.

25112890.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US/GNS	Serial No. 10/017,472
List of Patents and Publications for Applicant's	Applicant Sunil Chada *et al.*	
INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Filing Date: December 7, 2001	Group: 1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C63	Soo *et al.*, "Cutaneous rat wounds express C49a, a novel gene with homology to the human melanoma differentiation associated gene, Mda-7," *J. Cell. Biochem,* 74:1-10, 1999..
	C64	Stokke *et al.*, "Uncoupling of the order of the S and M phases: effects of staurosporine on human cell cycle kinases," *Cell Prolif.*, 30(5):197-218, 1997.
	C65	Su *et al.*, "The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice," *Proc. Nat'l Acad. Sci, USA,* 95: 14400-14405, 1998.
	C66	Templeton *et al.*, "Improved DNA: liposome complexes for increases systemic delivery and gene expression," *Nat. Biotechnol.*, 15:647-652, 1997.
	C67	Verma and Somia, "Gene therapy—promises, problems and prospects," *Nature*, 389:239-242, 1997.
	C68	Vogelstein and Kinzler, "p53 function and dysfunction," *Cell*, 70:523-526, 1992.
	C69	Walther and Stein, "Viral vectors for gene transfer: a review of their use in the treatment of human diseases," *Drugs*, 60:249-271, 2000.
	C70	White, "Life, death, and the pursuit of apoptosis," *Genes Dev.*, 10:1-15, 1996.
	C71	Zhang *et al.*, "Identification of a novel ligand-receptor pair constitutively activated by ras oncogenes," *J. Biol. Chem.*, 275:24436-24443, 2000

25112890.1

EXAMINER:	DATE CONSIDERED: 9-9-03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)	Atty. Docket No. INGN:097US	Serial No. 10/017,472
List of Patents and Publications for Applicant's INFORMATION DISCLOSURE STATEMENT (Use several sheets if necessary)	Applicant Sunil Chada *et al.*	
	Filing Date: December 7, 2001	Group: 1632

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 2*	*See Page 2*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
	A24	2002/091098	7-11-02	Fisher	514	44	11-21-01

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No
	B7	WO 00 71096	11-30-00	PCT			
	B8	WO 01 05437	2-25-01	PCT			

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation
	C72	Madireddi et al., "A novel melanoma differentiation associated gene with promise for cancer gene therapy," *Cancer Gene Therapy,* 465:239-261, 2000.
	C73	Ramesh et al., "Inhibition of lung tumor growth following adenovirus-mediated mda-7 gene expression in vivo," *Proc. Amer. Assoc. Canc. Res. Annual Meeting,* 42:657, 2001.
	C74	Saeki et al., "Tumor-suppressive effects by adenovirus-mediated mda-7 gene transfer in non-small cell lung cancer cell in vitro," *Gene Therapy,* 7:2051-2057, 2000.

25298911.1

EXAMINER:	DATE CONSIDERED: 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Form PTO-1449 (modified)

List of Patents and Publications for Applicant's

INFORMATION DISCLOSURE STATEMENT

(Use several sheets if necessary)

Atty. Docket No.	Serial No.
INGN:097US/GNS	10/017,472
Applicant	
Sunil Chada *et al.*	
Filing Date:	**Group:**
December 7, 2001	1645

U.S. Patent Documents	Foreign Patent Documents	Other Art
See Page 1	*See Page 1*	*See Page 1*

U.S. Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Name	Class	Sub Class	Filing Date of App.
	A21	5,643,761	7/1/97	Fisher *et al.*	435	91.1	10/27/93
	A22	5,710,137	1/20/98	Fisher	514	44	8/16/96
	A23	6,355,622 B1	3/12/02	Fisher	514	44	2/16/99

Foreign Patent Documents

Exam. Init.	Ref. Des.	Document Number	Date	Country	Class	Sub Class	Translation Yes/No

Other Art (Including Author, Title, Date Pertinent Pages, Etc.)

Exam. Init.	Ref. Des.	Citation

25166844.1

EXAMINER: _____ **DATE CONSIDERED:** 9/9/03

EXAMINER: INITIAL IF REFERENCE CONSIDERED, WHETHER OR NOT CITATION IS IN CONFORMANCE WITH MPEP609; DRAW LINE THROUGH CITATION IF NOT IN CONFORMANCE AND NOT CONSIDERED. INCLUDE COPY OF THIS FORM WITH NEXT COMMUNICATION TO APPLICANT.

Notice of References Cited

Application/Control No.	Applicant(s)/Patent Under Reexamination	
10/017,472	CHADA ET AL.	
Examiner	Art Unit	Page 1 of 2
Q. Janice Li	1632	

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-6,355,622	03-2002	Fisher, Paul B.	514/44
	B	US-6,069,134	05-2000	Roth et al.	514/44
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Deonarain, 1998, Expert Opin. Ther. Pat., Vol. 8, pages 53-69.
	V	Miller et al, 1995, FASEB J., Vol. 9, pages 190-199.
	W	Makrides et al, Protein Exp Pur 1999;17:183-202.
	X	Boucher et al, J Clin Invest 1999 Feb; 103:441-5.

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Notice of References Cited

Application/Control No.	Applicant(s)/Patent Under Reexamination	
10/017,472	CHADA ET AL.	
Examiner	Art Unit	Page 2 of 2
Q. Janice Li	1632	

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Bowie et al, Science 1990 Mar; 247:1306-10.
	V	Skolnick et al, TIBTECH 2000 Jan;18:34-9.
	W	Rudinger, Peptide Hormones 1976; June;pages 1-7.
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

EXHIBIT D

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450, on the date below:

January 30, 2004
Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

I. AMENDMENT; AND II. RESPONSE TO OFFICE ACTION DATED SEPTEMBER 30, 2003

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Commissioner:

This paper is submitted in response to the Office Action dated September 30, 2003 for which the three-month date for response was December 30, 2003.

A request for a one-month extension of time to respond is included herewith along with the required fee. This extension will bring the due date to January 30, 2004, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INGN:097US.

Amendments to the claims are reflected in the listing of claims, which begins on page 3 of this paper.

Remarks/Arguments in response to the Office Action begin on page 9 of this paper.

I. AMENDMENT

Listing of Claims

1. (Currently amended) A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis, ~~whereby the MDA-7 polypeptide inhibits angiogenesis in the patient~~.

2. (Original) The method of claim 1, wherein said patient exhibits an angiogenesis-related disease.

3. (Original) The method of claim 2, wherein the angiogenesis-related disease is further defined as angiogenesis-dependent cancer, a benign tumor, rheumatoid arthritis, psoriasis, an ocular angiogenic disease, Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, a telangiectasia, hemophiliac joint, angiofibroma, wound granulation, cat scratch disease, an ulcer, an intestinal adhesion, atherosclerosis, scleroderma, or a hypertrophic scar.

4. (Original) The method of claim 3, wherein angiogenesis-dependent cancer is further defined as a solid tumor, leukemia, or a tumor metastasis.

5. (Withdrawn) The method of claim 3, wherein the benign tumor is further defined as a hemangioma, a neuroma, a neurofibroma, a trachoma, uterine fibroid, hamartoma, teratoma, or a pyogenic granuloma.

6. (Withdrawn) The method of claim 3, wherein the ocular angiogenic disease is further defined as diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, or Rubeosis.

7. (Original) The method of claim 1, wherein the nucleic acid is an expression vector.

8. (Original) The method of claim 7, wherein the expression vector is a viral vector.

9. (Original) The method of claim 8, wherein the viral vector is administered at between 10^3 and 10^{13} pfu.

10. (Original) The method of claim 8, wherein said viral vector is an adenoviral vector, a retroviral vector, a vaccinia viral vector, an adeno-associated viral vector, a polyoma viral vector, or a herpesviral vector.

11. (Original) The method of claim 8, wherein said viral vector is an adenoviral vector.

12. (Original) The method of claim 1, wherein said nucleic acid further comprises a CMV IE, dectin-1, dectin-2, human CD11c, F4/80, SM22 or MHC class II promoter.

13. (Original) The method of claim 1, wherein the MDA-7 polypeptide or nucleic acid is administered to the patient by direct injection into an area in need of inhibition of angiogenesis.

14. (Original) The method of claim 13, wherein the patient is administered multiple injections.

15. (Currently amended) The method of claim [13] 1, wherein the injection is performed locally to a disease site.

16. (Currently amended) The method of claim [13] 1, wherein the injection is performed regionally to a disease site.

17. (Currently amended) The method of claim [13] 1, wherein the injection is performed distally to a disease site.

18. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by continuous infusion.

19. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered to the patient by intravenous injection.

20. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered prior to or after surgery.

21. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered before chemotherapy, immunotherapy, or radiotherapy.

22. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered during chemotherapy, immunotherapy, or radiotherapy.

23. (Original) The method of claim 1, wherein the MDA polypeptide or the nucleic acid is administered after chemotherapy, immunotherapy, or radiotherapy.

24. (Original) The method of claim 1, wherein the patient is a human.

25. (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

26.-31. (Cancelled)

32. (Original) The method of claim 1, wherein the MDA polypeptide comprises amino acids from 182 to 206 of SEQ ID NO:2.

33. (Original) The method of claim 1, wherein the MDA polypeptide comprises a secretory signal.

34. (Original) The method of claim 33, wherein the secretory signal is further defined as a positively charged N-terminal region in combination with a hydrophobic core.

35. (Original) The method of claim 1, wherein the patient is a cancer patient.

36. (Currently amended) A method of inhibiting endothelial cell differentiation in a human patient comprising administering to the patient an effective amount of a human MDA-7 polypeptide or a nucleic acid molecule expressing the human MDA-7 polypeptide.

37. (Original) The method of claim 36, wherein a chemotherapeutic agent is administered prior to administration of the MDA-7 polypeptide or the nucleic acid molecule.

38. (Original) The method of claim 36 wherein a chemotherapeutic agent is administered after administration of the MDA-7 polypeptide or the nucleic acid molecule.

39. (Original) The method of claim 36, wherein the chemotherapeutic agent is a DNA damaging agent.

40. (Original) The method of claim 39, wherein the DNA damaging agent is gamma-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP), or hydrogen peroxide.

41. (Original) The method of claim 38, wherein the chemotherapeutic agent is a cisplatin (CDDP), carboplatin, procarbazine, mechlorethamine, cyclophosphamide, camptothecin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, doxorubicin, bleomycin, plicomycin, mitomycin, etoposide (VP16), tamoxifen, taxol, transplatinum, 5-fluorouracil, vincristin, vinblastin, methotrexate, or analog or derivative variant thereof.

42. (Original) The method of claim 36, wherein the nucleic acid is comprised within a viral vector.

43. (Original) The method of claim 36, wherein the nucleic acid is comprised in a lipid composition.

68. (Withdrawn) The method of claim 32, wherein the MDA polypeptide comprises amino acids from 175 to 206 of SEQ ID NO:2.

69. (Withdrawn) The method of claim 68, wherein the MDA polypeptide comprises amino acids from 150 to 206 of SEQ ID NO:2.

70. (Withdrawn) The method of claim 69, wherein the MDA polypeptide comprises amino acids from 125 to 206 of SEQ ID NO:2.

71. (Withdrawn) The method of claim 70, wherein the MDA polypeptide comprises amino acids from about 100 to about 206 of SEQ ID NO:2.

72. (Withdrawn) The method of claim 71, wherein the MDA polypeptide comprises amino acids from 75 to 206 of SEQ ID NO:1.

73. (Withdrawn) The method of claim 72, wherein the MDA polypeptide comprises amino acids from 49 to 206 of SEQ ID NO:2.

74. (Withdrawn) The method of claim 73, wherein the MDA polypeptide comprises amino acids from 1 to 206 of SEQ ID NO:2.

75. (New) The method of claim 8, wherein 10^{10} to 10^{13} viral particles are administered.

76. (New) The method of claim 75, wherein 10^{11} to 10^{12} viral particles are administered.

77. (New) The method of claim 3, wherein the angiogenesis-dependent cancer is a hepatocarcinoma, retinoblastoma, astrocytoma, leukemia, neuroblastoma, mesothelioma, or non-small cell lung, small-cell lung, lung, head, neck, pancreatic, prostate, renal, bone, testicular, ovarian, cervical, gastrointestinal, lymphoma, brain, colon or bladder cancer.

II. RESPONSE TO OFFICE ACTION

A. Status of the Claims

Claims 1-25, 32-43, and 68-74 were pending prior to the Office Action dated September 30, 2003. Claims 1, 15-17, and 36 have been amended. Support for the amendments may be found throughout the specification, for example, at page 10, lines 7-10 and page 22, lines 21-25 and in the originally filed claims, such as claim 36. Claims 75-77 have been added. Support for these claims can be found in the specification, for example, at page 56, lines 27-28 and at page 9, lines 9-13, 18-20. No new matter has been added.

The withdrawal of claims 68-74 from consideration is inappropriate. Claim 32 contains the elected species and added claims 68-74 depend from claim 32 and further limit the elected species.

B. Claim Objections

1. Amendment of Claims Due to Election

The Action objects to claims 1, 13, 18-23, and 36-38 because they encompass more than one invention as defined in the restriction requirement (Paper No. 8). The Action contends the claims should be amended so that they read only upon the elected invention. Applicants respectfully traverse this objection.

The MPEP states, "The linking claims must be examined with the invention elected, and should any linking claim be allowed, the restriction requirement must be withdrawn." MPEP 809. Linking claims include "genus claims linking species claims." MPEP 809.03. Claims 1 and 36 are linking claims.

Applicants respectfully request that the examiner provide the authority or citation that requires an applicant to *amend* claims based merely on an election of invention.

2. Recitation of "MDA-7"

The Action objects to claim 1 because of its recitation of "MDA-7" and requires that the abbreviation be spelled out. Claim 1 now recites "Melanoma Differentiation Antigen-7 (MDA-7)."

3. Claims 16 and 17 Are Amended

The Action objected to claims 16 and 17 as being improperly dependent. Claim 16 has been amended to depend from claim 1 instead of claim 13. Similarly, claim 17 has been amended to depend from claim 1 as well.

C. Claims 1-4, 7-25, and 32-43 Are Enabled

The Action rejects claim 1-4, 7-25, and 32-43 under 35 U.S.C. §112, first paragraph, because the specification allegedly does not enable the claimed invention for the following aspects: (1) MDA-7 polypeptide fragments; (2) addition of a secretory signal on an MDA-7 polypeptide; (3) targeting of a nucleic acid to a target site to effect tumor killing. Applicants respectfully traverse this rejection.

1. MDA-7 Fragments Are Enabled

The Action contends that neither the specification, nor art of record, teaches a consensus region that is critical for the function of MDA-7 or the structural correlation of the polypeptide with its function for inhibiting the growth of tumor cells. It also contends that the art of protein chemistry is "one of the most unpredictable areas of biotechnology." It cites the references of Bowie *et al.* as teaching that certain positions in a protein sequence are critical to the three-dimensional structure/function relationship and that these regions can tolerate only conservative substitutions or none at all (page 1306, column 2). The Action also cites the reference of Skolnick *et al.* to support its conclusion that one cannot predictably extrapolate the teaching of the specification to the scope of the claims because the skilled artisan cannot envision the

detailed structure of fragments of SEQ ID NO:2 encompassed by these claims with the function of the fragments. Moreover, it states that determination of the effects of particular modifications and fragmentations are not predictable until they are actually made, citing the reference of Rudinger. For these reasons, the Action concludes that it would have required undue experimentation to practice the invention.

The Action's contention that the specification fails to provide a sufficient teaching for fragments of MDA-7 lacks merit. A closer look at the cited references does not support the Action's conclusion.

For example, the Action's citation to the Skolnick reference is taken out of context with respect to the issue at hand. The Action cites Skolnick for stating 1) "Sequence-based methods for function prediction are inadequate because of the multifunctional nature. However just knowing the structure of the protein is also insufficient for prediction of multiple functional sites" (abstract)"; and 2) "Knowing a protein's three-dimensional structure is insufficient to determine its function." The entire Skolnick paper is focused on the issue of *predicting* what a protein's function *might be* when *only* sequence information is available, such as in the context of genome sequencing-type projects, where cDNA sequences are obtained. This is reflected by the title of the reference, "From genes to protein structure and function: novel applications of computational approaches in the genomic era." The Skolnick reference might be relevant if Applicants were claiming a cDNA sequence for which no utility had been established. However, this reference is not relevant to the claimed invention because a function for MDA-7 is *already* provided and this is recited in the claims.

The Action contends that "Determination of the effects of particular modifications and fragmentations are not predictable until they are actually made and used, hence resulting in a trial

and error situation." Action at page 6. However, the standard for enablement is not the need for "trial and error." The test of enablement is whether the experimentation needed to practice the invention is undue. MPEP § 2164.01 (citing *Mineral Separation v. Hyde*, 242 U.S. 261, 270 (1916)). In fact, satisfaction of the enablement requirement is not precluded by the necessity of some experimentation. *See Atlas Powder Co. v. E.I. duPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409 (Fed. Cir. 1984). Therefore, even if trial and error were required to practice the invention, the Action has still not met its burden of showing that this is tantamount to requiring "undue experimentation."

The Action relies upon the reference of Rudinger to indicate that "painstaking experimental study" is required to predict the significance of particular amino acids and sequences for different aspects of biological activity. However, this reference is irrelevant to the present invention because it was published in 1976, ***almost 25*** years before the current application was filed. Surely this reference does not reflect the state of the art at the time the application was filed. Particularly notable is the fact that in the last 25 years, recombinant DNA technology has made something that was extremely difficult—requiring perhaps "painstaking experimental study"—25 years ago, such as cloning a gene, a trivial pursuit, as is demonstrated by the completion of the Human Genome Project in the last two years.

In fact, a skilled artisan could readily prepare fragments covered by the claims and test them for function. The specification provides the cDNA sequence for MDA-7 and teaches, for example, that fragments can be generated recombinantly. Specification at pages 35-44.

Applicants respectfully note that the PTO is required, when examining a patent application, to assume that the specification complies with §112 unless it has "acceptable evidence or reasoning" to suggest otherwise. *In re Marzocchi*, 439 F.2d 220, 223-24, 169 USPQ

367, 369-370 (CCPA. 1971). Thus, the PTO must provide reasons supported by the record as a whole what the specification is not enabling. *Application of Angstadt*, 537 F.2d 498, 504, 190 USPQ 214, 219-220 (CCPA 1979). Then and only then does the burden shift to the Applicants to prove that one of ordinary skill in the art could have practiced the claimed invention without undue experimentation. *In re Strahilevitz*, 668 F.2d. 1229, 1232, 212 USPQ 561, 563-64 (CCPA 1982). In this case, the cited references regarding protein sequence and structure do not shift the burden to the Applicants.

Moreover, Applicants provide additional evidence regarding an MDA-7 fragment. The Declaration of Sunil Chada ("Declaration") (Appendix A) indicates that an MDA-7 polypeptide lacking the first 48 amino acids of the full-length sequence induced cell killing in melanoma cells. Declaration at ¶ 6. Moreover, an MDA-7 polypeptide lacking the first 48 amino acids but containing a sequence targeting it to the endoplasmic reticulum suppressed the growth of prostate cancer cells (PC3 cells) and human non-small cell lung carcinoma cells (H1299 cells). Declaration at ¶ 7. Therefore, in view of the foregoing arguments, Applicants respectfully request this ground for the rejection be withdrawn.

2. Secretory Signal with MDA-7 Is Enabled

The Action contends that the reference of Su *et al.* teaches that the tumor suppressing effect of MDA-7 is associated with chromatin remodeling via its nucleus translocation from the cytosol and facilitating the migration of MDA-7 into the nucleus would enhance the selective growth inhibition of malignant but not normal cells. Therefore, the Action contends that the addition of a secretory signal on an MDA-7 polypeptide would prohibit the nucleus translocation and thus abolish the anti-tumor effect of MDA-7.

First, the Su reference cited by the Action does not provide data to indicate that addition of a secretory signal would abolish the anti-tumor effects of MDA-7; it merely speculates in the

Discussion section that facilitating migration of MDA-7 into the nucleus may enhance growth inhibition.

Moreover, the Declaration of Sunil Chada indicates that the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. The present specification indicates that there is a putative secretory signal in the first 46 amino acids of the protein. Specification at page 26, lines 1-9. Furthermore, the Declaration indicates that different forms of MDA-7 were evaluated in prostate cancer cells and human non-small cell lung carcinoma cells. An MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version) showed growth suppression in those cells, as did the full-length MDA-7. However, an MDA-7 targeted to the nucleus or an MDA-7 targeted to the cytoplasm did not. Also, higher levels of apoptosis were observed in cells transfected with full-length and ER versions of MDA-7, compared to the cytoplasmic or nuclear versions of MDA-7.

Therefore, there should not be any issues regarding whether an MDA-7 polypeptide with a signal sequence attached to it can achieve the anti-tumor effect of MDA-7.

3. Administration of MDA-7 Encoding Nucleic Acids Are Enabled

The Action generally contends that while progress has been made in recent years for gene transfer *in vivo*, targeting of naked nucleic acid or any vector to desired cells *in vivo* continues to be unpredictable and inefficient. While difficult to discern clearly, the Action seems to be making three points: 1) gene therapy is unpredictable; 2) targeting of a nucleic acid that is not a viral vector (nonviral vector-nucleic acid) is problematic; and 3) targeting of viral vectors, for example adenovirus, may be problematic.

i) Gene therapy

The Action contends that the gene therapy practitioner, while acknowledging the significant potential of gene therapy for cancer, still recognizes that such therapy was neither

routine nor accepted and await significant development and guidance for its practice. It cites the references of Miller *et al.*, Makrides *et al.*, and Boucher *et al.* to allegedly support its contention.

Once again, a closer examination of the cited references reveals that they do not support the Action's conclusions and also, there is evidence that indicates gene therapy can be practiced according to the specification and knowledge of the skilled artisan.

The Action cites the reference of Miller as saying, "No single delivery system is likely to be universally appropriate, for instance, the requirements of gene therapy for cystic fibrosis are greatly different from those of cancer." Action at page 8, citing page 190 of Miller. By its own admission, the Action renders the next citation to Miller and the citation to Boucher irrelevant because they both involve statements relating to the treatment of cystic fibrosis, while the present invention is related to inhibiting angiogenesis.

As for the reliance on the reference of Makrides, this reference merely states that "the choice of an expression system for production of recombinant proteins depends on many factors...." However, it is not clear how this statement indicates that undue experimentation would be required to practice the invention. Moreover, this reference says nothing about the ability to express MDA-7 or any limitations there might be with its expression.

In fact, there is evidence to support the contention that the claims are enabled. In addition to the data regarding a therapeutic effect from administration of Ad-mda7 in the specification (Examples 1, 4, 6, 9, 10 and 11), there is information relating to the administration of an MDA-7-encoding plasmid in a DOTAP:cholesterol liposome to a nude mouse. In the Declaration of Sunil Chada, he sets forth that nude mice with tumors exhibited reduced tumor growth and reduced levels of CD31 staining after treatment with the DOTAP:Chol-*mda-7*

complex. Declaration at ¶ 9. A reduction in levels of CD31 staining is indicative of reduced vascularization, *i.e.,* inhibition of angiogenesis.

ii) Nonviral-vector nucleic acids

To support its argument that gene therapy using a nonviral vector-nucleic acid, the Action refers to the reference of Deonarain. It cites Deonarain as stating that one of the biggest problems hampering successful gene therapy is the "ability to target a gene to a significant population of cells and express it at adequate levels for a long enough period of time." Action at page 7. The Action also says that the Deonarain reference gives high hope to targeted gene delivery, but that the strategies it discusses are still under investigation and that the reference concludes they were much less efficient than viral gene delivery.

However, there are several reasons the Deonarain reference does not support the broad conclusion that gene therapy with nonviral-vector nucleic acids is unpredictable and inefficient.

The Action's quotation from the first line of the abstract regarding "one of the main obstacles" to fulfilling the promise of gene therapy is taken out of context, because the reference goes on to say that "Viral methods of gene delivery have been studied for a number of years and are effective vectors for gene transfer." The Deonarain reference goes on to say that alternative methods are being explored because of issues relating to mutagenesis, side effects and toxicity—not targeting and expression levels.

Also, the conclusion that the Action cites from the Deonarain reference regarding targeted gene delivery being less efficient than viral gene delivery is followed by the statement, "However, under optimal conditions, enough gene product may be produced to give a therapeutic benefit (*e.g.*, suppress a phenotype of destroy a tumour)." Therefore, the use of the

specific nonviral vector nucleic acid delivery method discussed in the article is not plagued with as many problems as the Action contends.

Furthermore, this reference concerns specifically one type of nonviral vector nucleic acid delivery—"ligand-targeted receptor mediated vectors for gene delivery"—as the title indicates. There are other types of nonviral vector technology, which is not discussed by the Action at all. Therefore, even if one particular type of gene therapy is still undergoing experimentation and improvement, that does not mean that the instant claims reciting a "nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells" is not enabled.

Moreover, the concern regarding targeting and sustained expression of a gene may be less significant for a gene such as *mda-7*. As the specification indicates, MDA-7 induces apoptosis, and it selectively induces apoptosis in cancer cells, as opposed to normal cells. Specification at page 75. That MDA-7 induces apoptosis in a cell means expression of MDA-7 does not need to be sustained because once it enters the cell and induces apoptosis, that cell is no longer around. Additionally, because MDA-7 selectively induces apoptosis in cancer cells, targeting and sustained expression of MDA-7 are not the issues that they might be for many other gene therapies. Applicants contend that the Action does not raise provide credible reasons supported by the record for its contentions that undue experimentation would be required to practice the invention because it concerns gene therapy with nonviral vector-nucleic acids.

iii) Viral vectors

The Action also contends that it is not clear whether the recited vectors are suitable for the purpose of the instant invention. The Action contends that adenoviral vectors, for example, are known for their tissue tropism of respiratory epithelial cells, which would be a critical limitation for targeting any angiogenesis-dependent cancer.

First, the Action does not cite a reference or provide a declaration or affidavit to support this contention. Furthermore, the literature is replete with example of adenovirus infecting a variety of cell types, in addition to respiratory epithelial cells. In fact, the specification of the instant application shows that adenovirus infected breast cancer cells (Example 4), in addition to lung cancer cells (Example 10). Furthermore, the evidence cited above regarding clinical trials of tumor suppressors provides additional evidence that adenovirus can be used as a gene therapy vector, and that it is not limited to respiratory epithelial cells.

D. Claim 9 is Definite

The Action rejects claim 9 under 35 U.S.C. §112, second paragraph, as being indefinite for its recitation of "pfu." A random search in PubMed for articles published around the time the priority application for this application was filed reveals that "pfu" is used in the literature. Copies of two Journal of Virology papers are provided as examples to show that "pfu" is an appropriate unit dose (See abstracts) (Appendix B).

E. Claims Are Not Anticipated

1. Claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 Are Not Anticipated by Fisher

The Action rejects claims 1-4, 7, 8, 10-15, 24, 25, 35, 36, 42, and 43 under 35 U.S.C. §102(e) as being anticipated by Fisher (U. S. Patent No. 6, 355,622). Fisher is alleged to teach a method of inhibiting an angiogenesis-dependent cancer in a subject suffering from cancer comprising intratumoral administration of a replication-deficient adenoviral vector encoding the MDA-7 gene (amino acids 1-206 of SEQ ID NO:2) to nude mice bearing human cervical carcinoma cells. The Action contends that Fisher also teaches that the nucleic acid could be imbedded in liposomes introduced into the cell. Finally, it concludes that ectopic expression of MDA-7 inhibits the growth of tumor cells and may provide therapeutic benefit for the treatment

of human cancer, and as such, anticipates the instant claims. Applicants respectfully traverse this rejection.

The Federal Circuit case of *Kalman v. Kimberly-Clark Corp.*, 713 F.2d 760 (Fed. Cir. 1983) states that *identity of invention* is required for anticipation. *Each element* of the claim in issue must be found in a single prior art reference. The claims recite:

> A method of inhibiting angiogenesis in a human patient in need of such treatment comprising administering to the patient an effective amount of a human melanoma differentiation antigen-7 (MDA-7) polypeptide or a nucleic acid expressing the human MDA-7 polypeptide in eukaryotic cells to inhibit angiogenesis.

The Fisher patent, however, does not even mention angiogenesis or inhibition of angiogenesis. Accordingly, it does not anticipate the claimed invention. Applicants respectfully request this rejection be withdrawn.

2. Provisional Rejection Under 35 U.S.C. §102(e) of Claims 1-4, 7-25, and 35-43

The Action provisionally rejects 1-4, 7-25, and 35-43 under 35 U.S.C. §102(e) as being anticipated by copending application number 09/615,154, which has a common inventor with the instant application.

Because this rejection is provisional, Applicants will address this rejection, if necessary, once that application or the current application becomes otherwise allowable.

F. Claims 1, 7-9, 20-23, and 36-41 Are Not Obvious Over Roth *et al.* in View of Fisher

The Action rejects claims 1, 7-9, 20-23, and 36-41 under 35 U.S.C. §103(a) as being unpatentable over Roth *et al.* (U. S. Patent No. 6,069,134) in view of Fisher (U. S. Patent No. 6,355,622). It alleges that Roth teaches a method of administering a DNA damaging agent with an adenoviral vector expressing a tumor suppressor, particularly p53, for the treatment of cancer. The Action further contends that Fisher teaches using adenovirus encoding MDA-7 for the

treatment of cancer and administering vectors to tumor cells, which may provide a therapeutic benefit for the treatment of human cancer in general. The Action acknowledges that Fisher does not discuss the details of such therapy. The Action also argues that claims 20-23 and 37-41 have limitations regarding the timing of the combination therapy that neither of the references discusses. It alleges that these limitations, however, fall within the bounds of optimization for a proper therapeutic regimen that a person of ordinary skill in the art would know. It concludes that thus it would have been obvious to one of ordinary skill in the art at the time of the invention to modify the methods taught by Roth by simply substituting p53 with MDA-7 as taught by Fisher. The ordinary skilled artisan is alleged to have been motivated to modify the claimed invention because the combined therapy would maximize the tumor treating effect of any individual therapy alone. Applicants respectfully traverse this rejection.

Three basic criteria must be met to establish a *prima facie* case of obviousness:

(1) "there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings";
(2) "there must be a reasonable expectation of success"; and
(3) "the prior art reference (or references when combined) must teach or suggest all the claim limitations."

MPEP §2142. The present rejection does not meet at least two of these criteria because they do not teach or suggest all of the claim limitations and there was no reasonable expectation of success.

i) Claim limitations not taught by the combination of references

As discussed above, the Fisher patent does not mention angiogenesis. A review of the Roth patent reveals that it too does not mention angiogenesis. The claims recite inhibition of

angiogenesis and consequently, this combination of references does not teach each of the claim limitations.

ii) No reasonable expectation of success

The issue is whether the combination of references provided to the skilled artisan a reasonable expectation of achieving the claimed invention, which is inhibition of angiogenesis by administering a nucleic acid expressing the human MDA-7 polypeptide. As neither reference discusses angiogenesis, the skilled artisan would not have any reason to believe that combining the teachings of the references would provide a way to inhibit angiogenesis in a patient. Accordingly, the skilled artisan had no reasonable expectation of success with respect to the claimed invention. For this reason as well, a proper *prima facie* case is lacking. Applicants respectfully request this rejection be withdrawn.

G. Provisional Double Patenting Rejection

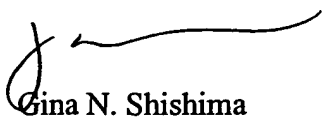
The Action provisionally rejects some of the claims of the application in view of copending U. S. Patent Application No. 09/615,154. Because this rejection is provisional, Applicants will, if necessary, address this rejection once claims in that application or the present application become otherwise allowable.

CONCLUSION

Applicants believe that the foregoing remarks fully respond to all outstanding matters for this application. Applicants respectfully request that the rejections of all claims be withdrawn so they may pass to issuance.

Should the Examiner desire to sustain any of the rejections discussed in relation to this Response, the courtesy of a telephonic conference between the Examiner, the Examiner's supervisor, and the undersigned attorney at 512-536-3081 is respectfully requested.

Respectfully submitted,



Gina N. Shishima
Reg. No. 45,104
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: January 30, 2004

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, Washington, DC 20231, on the date below:

January 3, 2003 — Date

Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Chada et al.

Serial No.: 10/017,472

Filed: December 7, 2001

For: METHODS OF TREATMENT INVOLVING HUMAN MDA-7

Group Art Unit: 1632

Examiner: Li, Qian J.

Atty. Dkt. No.: INGN:097US

DECLARATION OF SUNIL CHADA, Ph.D

I, Sunil Chada, declare:

1. I am the Director of Research and Development at Introgen Therapeutics. I have been working in the field of gene therapy and cancer biology for at least 15 years. My *curriculum vitae* is attached as Exhibit 1.

2. I am also one of the inventors named on the application identified above, which concerns the melanoma differentiation associated gene (mda-7) and its encoded protein, MDA-7.

3. The *mda-7* gene was first identified in human melanoma cell lines as a possible tumor suppressor. Jiang *et al.*, *Oncogene* 11:2477-86 (1995). Subsequent studies confirmed that elevated levels of MDA-7 suppressed cancer cell growth *in vitro* and selectively induced apoptosis in human breast cancer cells and inhibited tumorigenicity in nude

mice. Jiang *et al., Proc. Nat'l. Acad. Sci.* 93:9160-65 (1996); Su *et al., Proc. Nat'l Acad. Sci.* 95:14400-05 (1998).

4. I understand that the present application contains claims directed to methods of inhibiting angiogenesis involving administering a nucleic acid expressing the human MDA-7 polypeptide, which have been rejected as lacking enablement.

5. As described in this application, the first 48 amino acids of the full-length sequence may be cleaved to yield a secreted form of the protein. I have done scientific research on the tumor suppressor gene mda-7 and the MDA-7 protein, both the full-length and truncated versions.

6. In one study concerning the MDA-7 protein, human melanoma cell lines MeWo and WM35 were treated with increasing concentrations of an MDA-7 protein lacking the first 48 amino acids of the full-length sequence. The cell lines were analyzed in triplicate at 12, 24, 48, 72, and 96 hours after treatment using a trypan blue exclusion assay. This truncated MDA-7 protein induced cell killing in melanoma cells (Exhibit 2), but did not induce killing in lung cancer cells.

7. In another study, different forms of the MDA-7 protein were evaluated in PC3 human prostate cancer cells and H1299 human non-small cell lung carcinoma cells. The different forms (Exhibit 3) included: a full-length MDA-7, an MDA-7 protein lacking its own secretion signal (cytoplasmic version. lacking first 48 amino acids), an MDA-7 targeted to the nucleus (nuclear version), and an MDA-7 lacking its own secretion signal but containing a signal targeting it to the endoplasmic reticulum (ER version). Cells transfected with either the full-length or ER version of MDA-7 showed growth suppression (Exhibit 4). Furthermore, there were higher levels of apoptosis observed in

cells transfected with the full-length or ER versions, as compared to the cytoplasmic or nuclear versions of MDA-7.

8. Thus, as discussed in paragraph 6, the truncated version of MDA-7 does indeed induce apoptosis as set forth in the specification of this application. Furthermore, as discussed in paragraph 7, a truncated MDA-7 with a heterologous signal sequence suppresses growth and induces apoptosis.

9. Moreover, while the specification provides data regarding an Ad-mda7 construct to express MDA-7 in a eukaryotic cell, another study involved formulating a plasmid with an MDA-7 encoding nucleic acid in a liposome composition. The human mda-7 cDNA was placed under the control of the CMV promoter in a plasmid, which was formulated in a DOTAP:cholesterol complex. Nude mice were injected with human non-small cell lung carcinoma cells (A549 cell line) to produce tumors. Tumors were then treated intratumorally with the DOTAP:Chol-*mda-7* complex (50 μg/dose), resulting in the inhibition of tumor growth as compared to tumors in control animals. Similarly, tumors in nude mice from implantation of fibrosarcoma cells (UV223M cells) (syngeneic tumor model) were also inhibited by intratumoral administration of the DOTAP:Chol-*mda-7* complex. Moreover, when the tumor tissue from these animals were evaluated for CD31, they exhibited reduced levels of staining, which is indicative of reduced vascularization.

10. I hereby declare that all statements made of my own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such

willful false statements may jeopardize the validity of this application or any patent issued thereon.

01-29-04
Date

Sunil Chada, Ph.D.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
SUNIL CHADA	DIRECTOR OF RESEARCH AND DEVELOPMENT

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Kings College, University of London London, England	B.Sc. (Honors)	1982	Cell & Molecular Biology
University of California at Los Angeles Los Angeles, CA	M.Sc.	1985	Molecular Biology
University of Massachussetts Medical School Worcester, MA	Ph.D.	1988	Molecular Genetics

A.

B. **Positions and Honors.** List in chronological order previous positions, concluding with your present position. List any honors. Include present membership on any Federal Government public advisory committee.

PROFESSIONAL EXPERIENCE

1985-1988 Research Associate, Univ. of Massachusetts Medical School, Worcester MA
1988-1991 Research Scientist I, Dept. of Molecular Virology, Viagene Inc., San Diego CA
1991-1993 Research Scientist II, Dept. of Immunobiology, Viagene Inc., San Diego CA
1993-1995 Senior Scientist, Dept. of Immunobiology, Viagene Inc., San Diego CA
1995-1997 Staff Scientist, Chiron Technologies Inc., San Diego CA
1997-pres Director of Research and Development, Introgen Therapeutics, Houston TX
2002-pres Adjunct Faculty, Dept. of Bioimmunotherapy, Division of Cancer Medicine, MD Anderson Cancer Center

Committee Memberships

National Cancer Institute – SBIR/ STTR SRG Reviewer (standing member)
National Cancer Institute – Cancer Chemoprevention (Ad hoc member)
National Cancer Institute – RAID Committee member
Rice University – Advisory Board for NIH and NSF Biotechnology Training Programs
Alliance for Cancer Gene Therapy - Reviewer

C. **Selected peer-reviewed publications (from a total of 68).**

1) Chou C, Gatti RA, Fuller M, Concannon P, Wong A, **Chada S**, Davis R, and Salser W. "Structure and expression of ferritin genes in a human promyelocytic cell line that differentiates in vitro." *Molecular and Cellular Biology* 6:566-573 (1986).

2) Davis RC, Thomason AR, Fuller ML, Slovin JP, Chou CC, **Chada S**, Gatti RA, and Salser W. "mRNA species regulated during the differentiation of HL-60 cells to macrophages and neutrophils." *Developmental Biology* 119:164-174 (1987).

3) **Chada S**, Whitney C, and Newburger, PE. "Control of expression of the human glutathione peroxidase gene by selenium." In: *OxyRadicals in Molecular Biology and Pathology*, eds. Cerutti P, Fridovich I, and McCord J, UCLA Symposia on Molecular and Cellular Biology Vol. 82. Alan R. Liss, New York, pp.273-288 (1988).

4) Holland CA, **Chada S**, Wright J, Whitney C, Harigaya K, Greenberger J, Newburger PE. "Differentiation of Human Hematopoietic Cells Increases Expression of a Gene Transferred by a Retroviral Vector." *J. Leukocyte Biology* 46:221-229 (1989).

5) **Chada S**, Whitney C, and Newburger PE. "Post-transcriptional regulation of glutathione peroxidase gene expression by selenium in the HL-60 human myeloid cell line." *Blood* 74:2535-2541 (1989).

6) **Chada S** and Newburger PE. "Incorporation of selenocysteine at a UGA termination codon in the human glutathione peroxidase gene". In: *Glutathione Centennial; Molecular Perspectives and Clinical Implications*, eds. Taniguchi N, Higashi T, Sakamoto Y, and Meister A. Academic Press, Inc, San Diego pp:145-160 (1989).

7) **Chada S**, LeBeau M, and Newburger PE. "Isolation and chromosomal localization of the human glutathione peroxidase gene." *Genomics* 6:268-271 (1990).

8) Warner JF, Anderson CG, Laube L, Jolly D, Townsend K, **Chada S** and St. Louis D. "Induction of HIV-specific CTL and antibody responses in mice using retroviral vector transduced cells." *AIDS Research and Human Retroviruses* 7:645-655 (1991).

9) Jolly J, **Chada S**, Townsend K, DeJesus C, Chang S, Weinhold K, Anderson CG, Lynn A, Bodner M, Barber J, and Warner J. "CTL cross reactivity between HIV strains." *AIDS Research and Human Retroviruses* 8:1379-1381 (1992).

10) Warner JF, Anderson CG, Laube L, Jolly D, Irwin M and **Chada S**. "Immunotherapy using retroviral vectors" In "*Molecular Basis of Immune Responses*" eds. Nariuchi, H et al. Academic Press, Inc, San Diego. pp.155-165 (1993).

11) **Chada S**, DeJesus C, Townsend K, Lee W, Laube L, Jolly D, Chang S and Warner J. "Cross-reactive lysis of human targets infected with prototypic and clinical HIV-1 strains by murine anti HIV-1 IIIB env specific CTL." *Journal of Virology* 67:3409-3417 (1993)

12) Thor G, Sepulveda H, **Chada S** and Dutton RW. "A monoclonal antibody that distinguishes between a phosphorylated, beta-2 microglobulin associated and a non-phosphorylated, free heavy chain of MHC class I." *Journal of Immunology*.151:1-14 (1993)

13) Sajaddi N, Kamantigue E, Edwards W, Howard T, Jolly D and **Chada S**. "Recombinant retroviral vector delivered intramuscularly localizes to the site of injection in mice." *Human Gene Therapy* 5:693-699 (1994)

14) Irwin M, Laube L, Lee V, Austin M, **Chada S**, Anderson C-G, Townsend K, Jolly D and Warner J. "Direct injection of a recombinant retroviral vector induces HIV specific immune responses in mice and nonhuman primates". *Journal of Virology* 68:5036-5044 (1994)

15) Shen Q, **Chada S**, Whitney C and Newburger PEN. "Regulation of the human cellular glutathione peroxidase gene during monomyelocytic differentiation." *Blood* 84:3902-3908 (1994)

16) Newburger PEN, Malawista S, Dinauer M, Gelbart T, Woodman R, **Chada S**, Shen Q, von Blaricam G, Quie P and Curnutte J. "Chronic Granulomatous Disease and glutathione peroxidase deficiency, revisited" *Blood* 84:3861-3869 (1994)

17) Fouts T R, Tuskan R G, **Chada S**, Hone D and Lewis G K. "Construction and Immunogenicity of *S. typhimurium* Vaccine Vectors that express HIV-1 gp160." *Vaccine* 13: 1797-1705 (1996)

18) T. W. Dubensky, Driver DA, Polo JM, Belli BA, **Chada S**, Brumm D, Banks TA, Mento SJ, Jolly DJ and Chang SM. "Sindbis virus DNA-based expression vectors: utility for *in vitro* and *in vivo* gene transfer." *Journal of Virology* 70: 508-519 (1996)

19) Kamantigue E, Edwards W, **Chada S**, Brumm, D, Austin M, Irwin M, Mento S and Sajjadi N. "Evidence for localization of biologically active recombinant retroviral vector to lymph nodes and the site of injection in mice injected intramuscularly". *Gene Therapy* 3: 128-136 (1996)

20) Song ES, Lee V, Suhr CD, Brumm D, Lynn A, Jolly DJ, Warner JF, **Chada S**. "Mechanisms of retroviral mediated *in* vivo gene transfer and antigen presentation." *Proceedings of the National Academy of Sciences, U S A* 94:1943-1948 (1997)

21) Kline RM and **Chada S.** "Killing The Last Cell: Using The Immune System To Fight Cancer." *MIT Technology Review.* June:48-55 (1997)

22) Nguyen KHY, Boyle DL, McCormack JE, **Chada S** and Firestein G. "Direct synovial gene transfer with retroviral vectors in rat adjuvant arthritis". *Journal of Rheumatology* 25: 1118-1125 (1998)

23) Ishida T, **Chada S**, Stipanov M, Nadaf S, Ciernik FI, Gabrilovich DI and Carbone DP. "Dendritic cells transduced with wild type p53 gene elicit potent anti-tumor immune responses". *Clinical and Experimental Immunology.* 117(2):244-51 (1999)

24) Boyle DL,. Nguyen KHY, Zhaung S, McCormack JE, **Chada S**, and Firestein GS. "Intra-articular IL-4 gene therapy in arthritis: Clinical efficacy and enhanced Th2 activity". *Gene Therapy.* 6:1911-1918 (1999)

25) Russell JS, Lang FF, Huet T, Janicot M, **Chada S**, Wilson DR and Tofilon PJ. "Radiosensitization of human tumor cell lines induced by the adenovirus-mediated expression of an anti-ras single chain antibody fragment". *Cancer Research.* 59(20):5239-44 (1999)

26) Saeki T, Mhashilkar AM, Roth JA,, Branch C, **Chada S** and Ramesh R. "Tumor-suppressive effects by adenovirus-mediated *mda-7* gene transfer in non-small cell lung cancer cell *in vitro*". *Gene Therapy* 7: 2051-2057 (2000)

27) Mhashilkar AM, Schrock RD, Hindi M, Liao J, Sieger K, Kourouma F, Zou-Yang H, Onishi E, Takh O, Vedvick T, Fanger G, Stewart L, Watson GJ, Snary D, Fisher PB, Saeki T, Roth JA, Ramesh R and **Chada S.** "Melanoma-Differentiation Associated Gene (mda-7): A Novel Anti-Tumor Gene for Cancer Gene Therapy". *Molecular Medicine,* 7(4): 271-282 (2001). Cover article.

28) Nikitina EY, Clark JI, Benyen J, **Chada S**, Virmani AK, Carbone DP and Gabrilovich DI. "Generation of Anti-tumor Cytotoxic T Lymphocytes from Peripheral Blood of Cancer Patients using Dendritic cells transduced with Wild Type p53 Gene". *Clinical Cancer Research,* 7:127-135 (2001).

29) Saeki T, Mhashilkar AM, Swanson X, Zou-Yang H, Zumstein L, Sieger K, Branch CD, Roth JA, **Chada S** and Ramesh R. "Inhibition of human lung cancer growth following adenovirus-mediated *mda-7* gene expression *in vivo*". *Oncogene* 21(29): 4558-66 (2002)

30) Ekmekcioglu S, Ellerhorst J, Mhashilkar A, Sahin AA, Read CM, Prieto VG, **Chada S**, and Grimm EA. "Down-Regulation Of Melanoma Differentiation Associated Gene *(mda-7)* Expression In Human Melanomas" *International Journal of Cancer* 94: 54-59 (2001)

31) Huang EY, Madireddi MT, Gopalkrishnan R, Lesczyniecka M, Su ZZ, Lebedeva IV, Kang DC, Jiang H, Lin JJ, Alexandre D, Chen Y, Vozhilla N, Mei MX, Christansen KA, Sivo F, Goldstein N, Mhashilkar A, **Chada S**, Huberman E, Pestka S and Fisher PB. "Genomic Structure, chromosomal localization and expression profile of mda-7, a novel cancer-specific growth suppressing and apoptosis inducing gene". *Oncogene 20*: 7051-7063 (2001)

32) Ellerhorst JA, Prieto VG, Ekmekcioglu S, Broemeling L, Yekell S, **Chada S** and Grimm EA. "Loss of MDA-7 Expression with Progression of Melanoma". *Journal of Clinical Oncology* 20(4):1069-74 (2002)

33) Mohiuddin I, Cao X, Ozarvan MK, Zumstein LA, **Chada S**, Smythe WR. "PTEN Over-expression Engenders Cellular Death In Human Malignant Mesothelioma Cells Via Inhibition of AKT Phosphorylation". *Annals of Surgical Oncology.* 9(3):310-6 (2002)

34) Stewart AL, Mhashilkar AM, Yang X-H, Ekmekcioglu S, Saito Y, Sieger K, Schrock RD, Onishi E, Swanson X, Mumm JB, Zumstein L, Watson G, Snary D, Roth JA, Grimm EA, Ramesh R and **Chada S.** "PI3K blockade by Ad-PTEN Inhibits Invasion and Induces Apoptosis in Radial Growth Phase and Metastatic Melanoma Cells". *Molecular Medicine.* 8:451-462 (2002) Cover article

35) Caudell E, Mumm J, Poindexter N, Ekmekcioglu S, Mhashilkar A, Yang X-H, Sieger K, **Chada S** and Grimm EA. "MDA-7 is a Novel Th1 Cytokine that is Antagonized by IL-10". *Journal of Immunology* 168: 6041-6046 (2002)

36) Nikitina EY, **Chada S**, Muro-Cacho C, Fang B, Zhang R, Roth JA, and Gabrilovich DI. "An effective immunization and cancer treatment with activated dendritic cells transduced with full-length wild-type p53". *Gene Therapy.* 9(5):345-52 (2002)

37) Kawabe S, Nishikawa T, Munshi A, Roth JA, **Chada S** and Meyn R. "Adenovirus Mediated mda-7 Gene Expression Radiosensitizes Non-Small Lung Cancer Cells via p53-independent mechanisms". *Molecular Therapy.* 6(5): 637-644 (2002)

38) Pataer A, Vorburger SA, Barber G, **Chada S**, Mhashilkar A, Zhou-Yang H, Balachandran S, Roth JA, Hunt KK and Swisher SG. "mda-7 induces apoptosis via upregulation of the double-stranded RNA dependent kinase PKR". *Cancer Research* 62:2239-2243 (2002)

39) Chen J, **Chada S**, Mhashilkar AM and Miano JM. "MDA-7/IL-24 Selectively Inhibits Vascular Smooth Muscle Cell Growth and Migration". *Molecular Therapy.* Aug;8(2):220-9 (2003)

40) Pataer A, **Chada S**, Hunt KK, Roth JA and Swisher SG. "Adenoviral MDA-7 induces apoptosis in lung cancer cells through Mitochondrial Permeability Transition independent Cytochrome C Release". *Journal of Thoracic and Cardiovascular Surgery* 126(6):1328-1325 (2003)

41) Sasaki JI, Ramesh R, **Chada S**, Gomyo Y, Roth JA, and Mukhopadhyay T. "An Anthelminthic Drug to Curb Human Cancer: A Novel Microtubule Inhibitor as a Potential Inducer of Apoptosis in Non-Small Cell Lung Cancer Cells but Not in Normal Cells *In Vitro* and *In Vivo*. *Molecular Cancer Therapeutics.* 1: 1201-1209 (2002)

42) Ekmekcioglu S, Ellerhorst JA, Mumm JB, Zheng M, Broemeling L, Prieto VG, Stewart AL, Mhashilkar AM, **Chada S** and Grimm EA. "Negative Association of Melanoma Differentiation Associated Gene (mda-7) and inducible Nitric Oxide Synthase (iNOS) in Human Melanoma: MDA-7 Regulates iNOS Expression in Melanoma Cells." *Molecular Cancer Therapeutics.* 2(1):9-17 (2003)

43) Ramesh R, Mhashilkar A, Tanaka F, Saito Y, Sieger K, Mumm JB, Stewart A, Branch CD, Boquio A, Doumoutier L, Kotenko S, Roth JA and **Chada S**. " MDA-7/ IL-24 is a novel ligand that regulates angiogenesis via the IL-22 receptor". *Cancer Research.* 63(16):5105-13. (2003)

44) Mhashilkar AM, Stewart A, Sieger K, Ito I, Saito Y, Roth JA, Ramesh R and **Chada S**. "MDA-7 Negatively Regulates the beta-Catenin and PI3K Signaling Pathways in Breast and Lung Tumor Cells". *Molecular Therapy,* 8(2):207-19 (2003)

45) **Chada S**, Mhashilkar A and Gabrilovich D. "Development of vaccines against self antigens: the p53 paradigm". *Current Opinion in Drug Discovery and Development.* Mar;6(2):169-73 (2003)

46) Fisher PB, Gopalkrishnan RV, **Chada S**, Ramesh R, Grimm EA, Rosenfeld MR, Curiel DT and Dent P. "*mda-7*/IL-24, a novel cancer selective apoptosis inducing cytokine gene". *Cancer Biology and Therapy*: 2(4):24-38 (2003)

47) Saito Y, Gopalan B, Mhashilkar AM, Roth JA, **Chada S**, Zumstein L, Ramesh R. Adenovirus-mediated PTEN treatment combined with caffeine produces a synergistic therapeutic effect in colorectal cancer cells. *Cancer Gene Therapy*; 10(11):803-13 (2003).

48) Saito Y, Swanson X, Mhashilkar AM, Oida Y, Schrock R, Branch CD, **Chada S**, Zumstein L, Ramesh R. Adenovirus-mediated transfer of the PTEN gene inhibits human colorectal cancer growth in vitro and in vivo. *Gene Therapy* ;10(23):1961-9 (2003)

49) **Chada S**, Ramesh R, Mhashilkar AM. Cytokine- and chemokine-based gene therapy for cancer *Curr Opin Mol Ther.* 5(5):463-74 (2003). .

50) Sieger KA, Mhashilkar AM, Stewart A, Yang H-Y, Mumm JB, Ramesh R and **Chada S.** "The tumor suppressor activity of MDA-7/IL-24 is mediated by intracellular protein in NSCLC cells". *Molecular Therapy* In Press (2004)

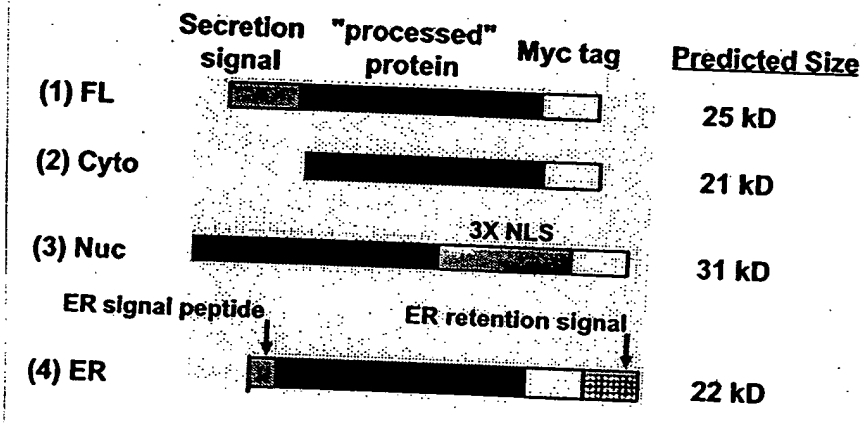
51) **Chada S**, Ekmekcioglu S, Ellerhorst JA, Mumm JB, Sahin A, Hunt KK, Ramesh R, Grimm EA and Mhashilkar AM. MDA-7/IL-24 is a unique cytokine in the IL-10 family". *International Immunopharmacology.* In Press (2004)

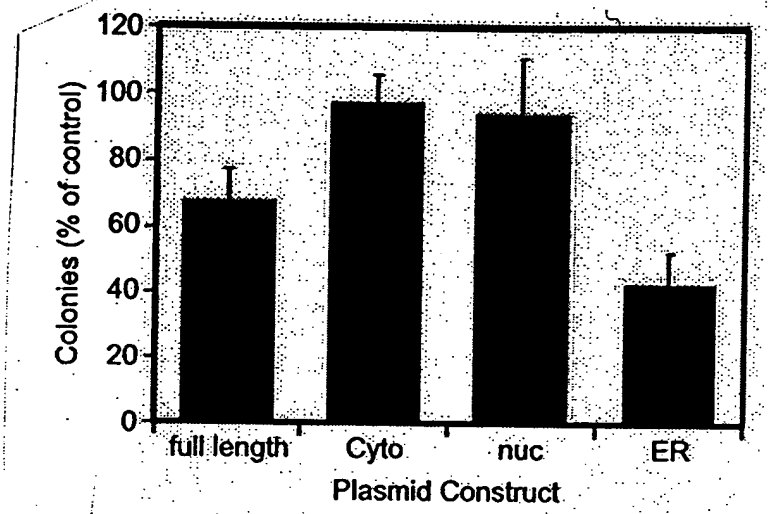
C. Research Support.

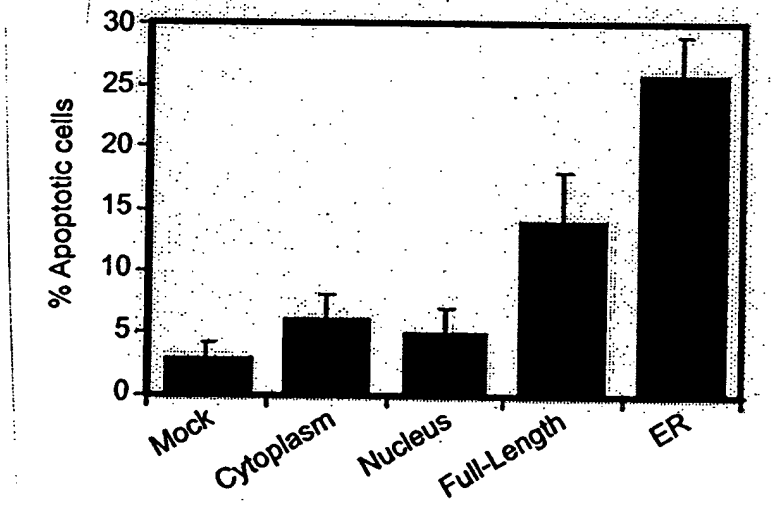
GRANTS AWARDED (from a total of 9)

1. Chada S. "Novel Gene Therapeutic for the Treatment of Lung Cancer" SBIR Grant 1R43CA86587-01 (Funded 03/00). Role: PI. Goals: to evaluate Ad-mda7 as a potential therapeutic for NSCLC
2. Meyn R "Tumor cell radiosensitization by gene drugs" STTR Grant. (Funded 08/00). Role: co-PI. Goal: to evaluate radiosensitization by Ad-p16 and Ad-mda7
3. Grimm EA "Novel gene therapy for Melanoma" STTR grant (Funded 06/01). Role: co-PI. Goals: to evaluate Ad--mda7 as a potential therapeutic for melanoma.
4. Chada S "Combination treatment for breast cancer using Ad-mda7 plus Herceptin". SBIR grant (Funded 07/02). Role: PI. Goals: To evaluate synergy between Ad-mda7 and Herceptin in breast cancer
5. Grimm EA "Phase II clinical trial for Melanoma using INGN 241 (Ad-mda7)" STTR grant (Funded 09/03). Role: co-PI

PATENTS and APPLICATIONS 8 issued patents; 17 applications pending


Secretion signal
"processed" protein
Myc tag
Predicted Size
(1) FL
25 kD
(2) Cyto
21 kD
3X NLS
(3) Nuc
31 kD
ER signal peptide
ER retention signal
(4) ER
22 kD


120
100
80
60
40
20
0
Colonies (% of control)
full length
Cyto
nuc
ER
Plasmid Construct


30
25
20
15
10
5
0
% Apoptotic cells
Mock
Cytoplasm
Nucleus
Full-Length
ER

JOURNAL OF VIROLOGY, May 2000, p. 4244–4252
0022-538X/00/$04.00+0

Vol. 74, No. 9

A Single Intramuscular Injection of Recombinant Plasmid DNA Induces Protective Immunity and Prevents Japanese Encephalitis in Mice

GWONG-JEN J. CHANG,* ANN R. HUNT, AND BRENT DAVIS

Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado 80522

Received 10 November 1999/Accepted 1 February 2000

Plasmid vectors containing Japanese encephalitis virus (JEV) premembrane (prM) and envelope (E) genes were constructed that expressed prM and E proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50,000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

Japanese encephalitis (JE) is a mosquito-borne viral disease of major public health importance in Asia. More than 35,000 cases and 10,000 deaths are reported annually (52). *Japanese encephalitis virus* (JEV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. More than 70 species in the *Flavivirus* genus have been genetically and serologically classified (29). Other important human pathogenic flaviviruses include yellow fever, dengue type 1 to 4 (DEN1 to DEN4), tick-borne encephalitis (TBE), and St. Louis encephalitis (SLE) viruses. Vaccination has been an effective mechanism for prevention of flavivirus infection in humans and domestic animals. Three JEV vaccines are in widespread production and use (52). These are inactivated virus from infected mouse brain, inactivated virus from primary hamster kidney cells, and a live attenuated SA14-14-2 vaccine. Only inactivated JEV vaccine, JEVAX, produced in mouse brain is distributed commercially and available internationally (52). Inactivated, mouse brain-derived whole virus vaccine is costly to prepare and carries the risk of allergic reaction to murine encephalitogenic basic proteins or gelatin stabilizer (45; M. M. Andersen, and T. Ronne, Letter, Lancet 337:1044, 1991). Since 1989, an unusual number of systemic reactions characterized by generalized urticaria and/or angioedema following JEVAX immunization have been reported from Australia, Canada, and Denmark (36). A major problem associated with use of the inactivated mouse brain vaccine is the failure to stimulate long-term immunity (39). Multiple immunization is recommended to provide adequate protection (28, 39). The attenuated JEV vaccine, SA14-14-2, is undergoing clinical trials (31). However, because of regulatory issues this vaccine has not found wide acceptance outside the People's Republic of China (11).

* Corresponding author. Mailing address: P.O. Box 2087, Division of Vector-Borne Infectious Diseases, CDC, Foothill Campus, Fort Collins, CO 80522-2087. Phone: (970) 221-6497. Fax: (970) 221-6476. E-mail: gxc7@cdc.gov.

Several experimental recombinant virus, attenuated virus, and subunit JEV vaccines have been reported. Recombinant baculovirus vector that contained the JEV envelope (E) protein gene has been used to infect insect cells and produce E protein that has been studied as a biosynthetic immunogen (33). Recombinant vaccinia viruses expressing the JEV genes extending from premembrane (prM) to NS2B proteins have been the most promising candidate vaccines. These candidate vaccines produced extracellular virus-like particles (EPs) in infected cell culture that induced high titers of neutralizing and hemagglutination-inhibiting antibodies and protective immunity in mice (19–21, 47, 54). Recombinant vaccinia viruses expressing the same JEV genes based on the attenuated vaccinia virus strain, NYVAC-JEV, or canarypox, ALVAC-JEV, were tested in phase I human trials (18). In this trial, only 1 in 10 ALVAC-JEV recipients developed detectable viral neutralizing antibody, and vaccinia virus-preimmune recipients had a significantly lower humoral immune response.

Inoculation of animals with purified plasmid vectors (DNA) by the intramuscular (i.m.) or intradermal route leads to expression of the recombinant vector-encoded protein in transfected cells, resulting in stimulation of a protein-specific immune response. Plasmid DNA vaccines provide an alternative to attenuated, inactivated, or virus-vectored subunit vaccines. Flavivirus DNA vaccines for Murray Valley encephalitis, DEN2, JE, SLE, and TBE (Central European encephalitis and Russian spring summer encephalitis) viruses have been developed and tested in the mouse model (4, 17, 24, 30, 38, 49). All of these plasmid DNA constructs contained similar transcriptional regulatory elements and a flavivirus gene cassette. Vaccination of mice with these plasmid DNA vaccines induced a virus-specific antibody response, as detected by enzyme-linked immunosorbent assay (ELISA). However, production of neutralizing antibody leading to 100% protection of vaccinated animals from virus challenge was observed only after multiple immunizations or delivery of DNA to the epidermis by particle

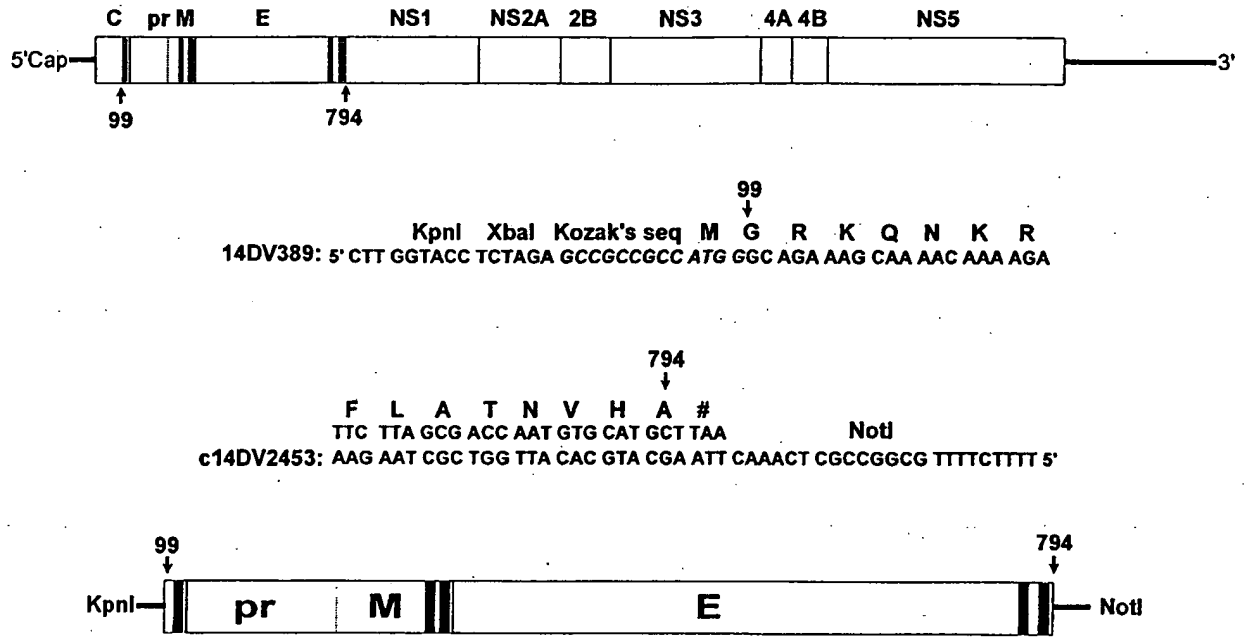


FIG. 1. Map of the JEV genomic structure (top) and the DNA sequence of oligonucleotides used in RT-PCR to construct the transcription unit for the expression of prM-E protein coding regions (bottom). Potential transmembrane helices of viral polyprotein are indicated by blackened areas.

bombardment (4, 24, 49). In this study, we constructed a JEV prM and E gene cassette that incorporates an extended signal peptide sequence at the NH_2 terminus of the prM gene and Kozak's sequence, an optimal translation enhancing element surrounding the AUG site. JEV protein expression was characterized using six different recombinant vectors containing the same insert. The humoral immune response and protection from virulent JEV challenge following immunization with the recombinant plasmid DNAs were compared to findings for the human vaccine, JEVAX, licensed by the U.S. Food and Drug Administration, in outbred ICR mice.

MATERIALS AND METHODS

Cell culture and virus strain. COS-1, COS-7, and SV-T2 cells (1650-CRL, 1651-CRL, and 163.1-CCL; American Type Culture Collection) were grown at 37°C in Dulbecco's modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 7.5% $NaHCO_3$ (30 ml/liter), penicillin (100 U/ml), streptomycin (100 μg/ml). COS-1 and COS-7 cells were derived from simian virus 40 (SV40) transformed CV1 cells which have an African green monkey kidney cell origin. SV-T2 cells were derived from SV40-transformed mouse fibroblasts. Vero cells were grown under the same conditions except that 5% fetal calf serum without nonessential amino acid was used. C6/36 cells (13) were grown at 28°C in the same medium used for the COS-1 cells. The SA14 strain of JEV, propagated by intracranial inoculation into suckling mouse brain, was used for animal challenges and plaque reduction neutralization tests (PRNT). The SA14 virus used in ELISA and Western blot experiments was propagated in C6/36 cells and purified by ultracentrifugation on 30% glycerol–45% potassium tartrate gradients (37).

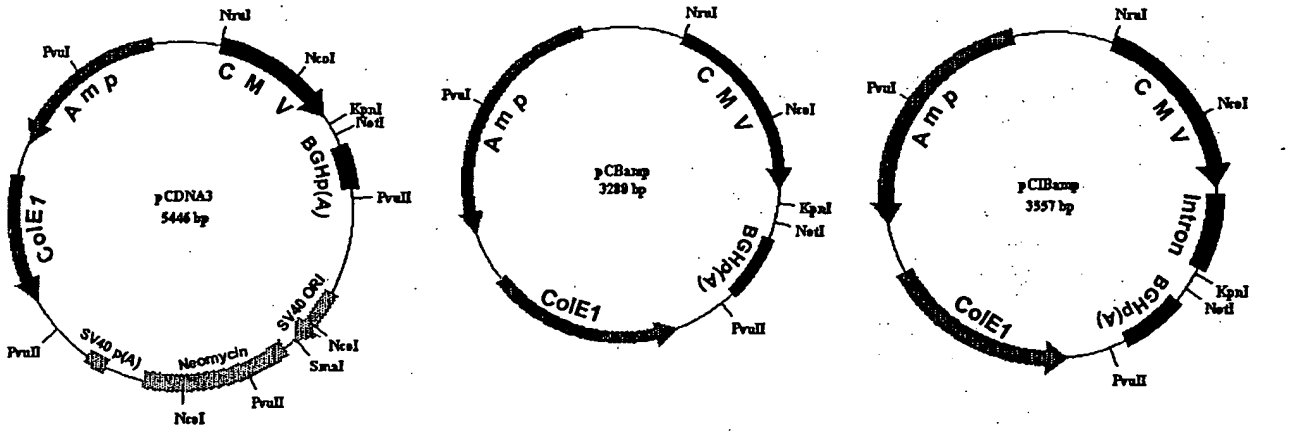
Construction of plasmids expressing JEV prM and E gene proteins. Genomic RNA was extracted from 150 μl of SA14 mouse brain JEV by using a QIAamp viral RNA kit (Qiagen, Santa Clarita, Calif.). RNA was adsorbed on a silica membrane, eluted in 80 μl of diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.)-treated water, and used as a template for amplification of JEV prM and E genes. Primer sequences were obtained from the published data (35). A single cDNA fragment containing genomic nucleotides (nt) 389 to 2478 was amplified by reverse transcriptase-mediated PCR (RT-PCR). Restriction enzyme sites for *KpnI* and *XbaI* and Kozak's sequence for an optimal translation initiation (25, 26) were engineered at the 5′ terminus of the cDNA by amplimer 14DV389. An in-frame translation termination codon, followed by a *NotI* restriction site, was introduced at the 3′ terminus of the cDNA by amplimer c14DV2453 (Fig. 1). A single-tube RT-PCR was performed using a Titan RT-PCR Kit (Roche Molecular Biochemical, Indianapolis, Ind.). The RT-PCR product was purified using a QIAquick PCR purification kit (Qiagen), and the DNA was eluted with 50 μl of 1 mM Tris-HCl (pH 7.5).

All vector constructions and analyses were carried out using standard techniques (46). RT-PCR-amplified cDNA was digested with enzymes *KpnI* and *NotI* and inserted into the *KpnI-NotI* site of eukaryotic expression plasmid vector pCDNA3 (Invitrogen, Carlsbad, Calif.). Electroportion-competent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, Calif.) were transformed by electroporation (Gene Pulser; Bio-Rad Laboratories, Hercules, Calif.) and plated on Luria broth (LB) agar plates that contained carbenicillin (100 μg/ml; Sigma). Clones were picked and inoculated into 3 ml of LB containing carbenicillin (100 μg/ml). Plasmid DNA was extracted from a 14-h LB culture by using a QIAprep Spin Miniprep kit (Qiagen). Automated DNA sequencing was performed as recommended on an ABI Prism 377 DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, Calif.). Both strands of the cDNA were sequenced and compared to the published SA14 virus sequence (35).

The pCDNA3 fragment from nt 1289 to nt 3455, which contained the f1-encoded eukaryotic origin of replication (ori), SV40 ori, neomycin coding region, and SV40 poly(A) elements, was deleted by *PvuII* digestion and then self-ligated to generate plasmid pCBamp. The pCIBamp vector, which contained a chimeric intron insertion at the *NcoI-KpnI* site of the pCB vector, was constructed by excising the intron sequence from pCI (Promega, Madison, Wis.) by digestion with *NcoI* and *KpnI*. The resulting 566-bp fragment was cloned into *NcoI-KpnI*-digested pCBamp to replace its 289-bp fragment. Figure 2 shows a schematic drawing of plasmids pCDNA3, pCBamp, and pCIBamp.

The DNA fragment containing the JEV coding region in the recombinant plasmid pCDJE2-7, derived from the pCDNA3 vector, was excised by *NotI* and *KpnI* or *XbaI* digestion and cloned into the *KpnI-NotI* sites of pCB, pCIB, pCEP4 (Invitrogen), and pREP4 (Invitrogen) and into the *SpeI-NotI* site of the pRc/RSV (Invitrogen) expression vector to create pCBJE1-14, pCIBJES14, pCEJE, pREJE, and pRCJE, respectively. Both strands of the cDNA from each plasmid vector were sequenced, and recombinant clones with a correct nucleotide sequence were identified. Plasmid DNA for in vitro transformation or mouse immunization was purified by anion-exchange chromatography using an Endo-Free Plasmid Maxi kit (Qiagen).

IFA. Expression of JEV-specific gene products by the various recombinant expression plasmids was evaluated by indirect immunofluorescence antibody assay (IFA) in the transient expression system using COS-1, COS-7, and SV-T2 cells. For transformation, cells were grown to 75% confluence in 150-cm^2 culture flasks, trypsinized, and resuspended in 4°C phosphate-buffered saline (PBS) to a final density of 1×10^7 to 2×10^7 cells/ml. Five hundred microliters of cell suspension was then electroporated with 10 μg of plasmid DNA, using a Bio-Rad Gene Pulser II set at 250 V and 960 μF. Cells were diluted with 25 ml of fresh medium after electroporation and seeded into one 75-cm^2 flask. Forty-eight hours after transformation, the medium was removed, and the cells were



TATA box KpnI BamHI EcoRI EcoRV NotI

TATATAA...(92nt)...*GGTACC*GAGCTC*GGATCC*ACTAGTAACGGCCGCCAGTGTGCTG*GAATTC*TGCA*GATATC*CATCACACTG*GCGGCCGC*...BHG-poly(A)

FIG. 2. Schematic representations of plasmid vectors pCDNA3, pCBamp, and pCIBamp. These plasmids include the CMV promoter/enhancer element, BGH poly(A) signal and transcription termination sequence [BHGp(A)], ampicillin resistance gene (Amp), and ColE1 ori for selection and maintenance in *E. coli*. The f1 ori for single-stranded rescue in *E. coli* cells, SV40 ori, neomycin coding region, and SV40 poly(A) [SV40 p(A)] sequences were deleted from pCDNA3 to generate pCBamp. An intron sequence was inserted in the *NcoI-KpnI* site of pCBamp to generate pCIBamp. The multiple cloning site for the insertion of JEV genes, located between the TATA box of the CMV promoter/enhancer and BHG poly(A) site, is shown.

trysinized and resuspended in 5 ml of PBS with 3% normal goat serum. Ten-microliter aliquots of the cell suspension were then spotted onto slides, air dried, and fixed with acetone at 4°C for 10 min. Immunofluorescent mapping of the E protein-specific epitopes was performed using a panel of murine monoclonal antibodies (MAbs) (15, 42, 55) and JEV-specific hyperimmune mouse ascitic fluid (HIAF). All antibodies were tested at 1:400 dilution in PBS.

Selection of an in vitro-transformed stable cell line constitutively expressing JEV-specific gene products. COS-1 cells transformed with 10 μg of pCDJE2-7 DNA by electroporation were incubated in nonselective culture medium for 24 h and then treated with neomycin (G418; 0.5 mg/ml; Sigma). G418-resistant colonies, which became visible after 2 to 3 weeks, were cloned by limited dilution in G418-containing medium. Expression of the JEV proteins was determined by IFA using JEV HIAF. One IFA-positive (JE-4B) and one IFA-negative (JE-5A) clone were selected for further analysis and maintained in medium containing 200 μg of G418 per ml. These stably transformed cells secreted antigen in the form of EPs (A. Hunt and G. J. Chang, unpublished data).

Antigen capture ELISA for detection of E protein secreted into culture fluid. The antigen capture ELISA, a modification of the procedure described by Guirakhoo et al. (8), was used to detect E protein from transiently transformed cells or JE-4B culture fluid. Flavivirus group-reactive MAb 4G2 was used to capture the JEV antigens (7). The 4G2-captured antigen was detected using horseradish peroxidase-conjugated MAb 6B6C-1 by incubation for 1 h at 37°C. Enzyme activity on the solid phase was detected with 3,3′,5,5′-tetramethylbenzidine ELISA substrate (Life Technologies, Grand Island, N.Y.); the reaction was stopped with the addition of 2 M H_2SO_4, and the optical density was measured at 450 nm.

Mouse experiments. Three-day-old mixed-sex or 3-week-old female ICR outbred mice were vaccinated i.m. with 50 or 100 μg of plasmid DNA at a concentration of 1 μg/μl in PBS or subcutaneously (s.c.) with 1/10 or 1/5 of the adult human dose of JEVAX (manufactured by the Research Foundation for Microbial Disease of Osaka University and distributed by Connaught Laboratories, Swiftwater, Pa.). The chloramphenicol acetyltransferase (CAT) protein expression plasmid pCDNA3/CAT (Invitrogen) was used as the vaccination control. Selected groups of mice were boosted 3 weeks later with an additional dose of plasmid vaccine or JEVAX. Mice were bled from the retro-orbital sinus; serum samples were evaluated for JEV antibody by ELISA and Western blotting using purified JEV and by PRNT.

Mice vaccinated at 3 days of age were challenged intraperitoneally (i.p.) 7 weeks postvaccination with JEV strain SA14 (50,000 PFU/100 μl) and observed for 3 weeks. To evaluate passive protection by maternal antibody, pups were obtained from mating of nonimmunized males with immunized females 9 weeks following their vaccination with plasmid DNA at 3 weeks of age. Pups were challenged by the i.p. route 3 to 15 days after birth with SA14 virus (5,000 PFU/100 μl) and observed daily for 3 weeks. Postchallenge serum was collected from survivors and tested for reactivity with JEV antigens by ELISA and Western blotting.

Serological tests. Postvaccination and postchallenge serum samples were tested for the ability to bind to purified JEV by ELISA, neutralize JEV infectivity by PRNT, or recognize JEV proteins by Western blotting (12, 41, 48). The PRNT assay was performed by incubating ~200 PFU of SA14 virus in 100 μl of Dulbecco's modified Eagle medium containing 5% bovine serum albumin and 20 mM HEPES buffer (pH 8.0) with serial twofold dilutions of serum specimens, started at 1:10, in 100 μl of the same buffer in 96-well trays at 4°C overnight. Serum specimens were heat inactivated at 56°C for 30 min before use. Duplicate 100-μl aliquots were assayed for infective virus by plaque formation on Vero cell monolayers. The percent plaque reduction was calculated relative to virus controls without serum. Titers were expressed as the reciprocal of serum dilutions yielding a 90% reduction in plaque number ($PRNT_{90}$).

RESULTS

Effect of the promoter and poly(A) signal on the efficiency of JEV prM and E protein expression. Four eukaryotic cell expression plasmids that contained the JEV coding region extending from genomic nt 390 to nt 2478 were constructed. This region of the genome encoded the prM and E genes. The Kozak sequence for the eukaryotic translation initiation site (underlined) of −9 to +4, GCCGCCGCCATGG, at the 5′ terminus (2, 25, 26, 27) and the in-frame translation terminat ion sequence at the 3′ terminus of cDNA were incorporated directly into cDNA by RT-PCR using viral RNA as a template. Transcription of the JEV genes in plasmid pCDJE2-7 was controlled by the human cytomegalovirus (CMV) early IA gene promoter/enhancer. The resulting mRNA is terminated and stabilized by a bovine growth hormone (BGH) transcript ion terminator and a poly(A) signal, respectively. The transcriptional control elements in pREJE were replaced by the Rous sarcoma virus (RSV) long terminal repeat promoter and SV40 poly(A). The pCEJE and pRCJE plasmids contain CMV plus SV40 poly(A) and RSV plus BGH poly(A), respectively (Table 1).

To determine the influence of the promoter and poly(A) elements on JEV prM and E protein expression, recombinant plasmids pCDJE2-7, pCEJE, pRCJE, and pREJE were ini-

TABLE 1. Transient expression of JEV prM and E proteins by various recombinant plasmids in two transformed cell lines

Name	Promoter	Intron	Poly(A)	Ori	Recombinant plasmid	IFA intensity/% positive[a]	
						COS-1	COS-7
pCDNA3	CMV	No	BGH	SV40	pCDJE2-7	3+/40	3+/35
pCBamp	CMV	No	BGH	No	pCBJE1-14	3+/45	ND
pCIBamp	CMV	Yes	BGH	No	pCIBJES14	3+/39	ND
pCEP4	CMV	No	SV40	OriP	pCEJE	2+/4	2+/3
pREP4	RSV	No	SV40	OriP	pREJE	1+/3	1+/2
pRc/RSV	RSV	No	BGH	SV40	pRCJE	1+/3	1+/3
pCDNA3	CMV	No	BGH	SV40	pCDNA3/CAT	−	−

[a] Various cell lines were transformed with pCDNA3/CAT (negative control), pCDJE2-7, pCBJE1-14, pCIBJES14, pCEJE, pREJE, or pRCJE. Cells were trypsinized 48 h later and tested by IFA with JEV HIAF. Data are presented as the intensity (scale of 1+ to 4+) and percentage of IFA-positive cells. pCDNA3/CAT-transformed cells were used as the negative control. ND, not determined. −, negative.

tially tested for the ability to express JEV prM and E proteins following transformation of various mammalian cells. COS-1, COS-7, and SV-T2 cells were transiently transformed with equal amounts of pCDJE2-7, pCEJE, pRCJE, or pREJE plasmid DNA. The SV-T2 cell line was excluded from further testing after preliminary results showed that less than 1% of pCDJE2-7-transformed SV-T2 cells were expressing JEV antigen.

JEV antigens were expressed in COS-1 and COS-7 cells transformed by all four recombinant plasmids, thus confirming that the CMV or RSV promoter and BGH or SV40 poly(A) elements were functionally active. However, the percentage of transformed cells and the level of JEV antigens expressed, as determined by the number of IFA-positive cells and IFA intensity, respectively, differed significantly (Table 1). A significantly higher percentage of pCDJE2-7-transformed COS-1 cells expressed JEV proteins with greater IFA intensity at a level equal to that observed with JEV-infected cells. Cells transformed with the pCEJE, pREJE, or pRCJE vector, on the other hand, showed a lower percentage of antigen-expressing cells as well as a lower IFA intensity. Vectors containing the CMV promoter and BGH poly(A) were selected for further analysis (Fig. 2).

To determine whether the enhanced expression of JEV proteins by the pCDJE2-7 vector was influenced by the SV40 ori, we constructed the pCBJE1-14 vector in which a 2,166-bp fragment containing the f1 ori, SV40 ori, neomycin coding region, and SV40 poly(A) elements was deleted. A chimeric intron was then inserted into pCBJE1-14 to generate pCIBJES14. Plasmid pCIBJES14 was used to determine whether the expression of JEV proteins could be enhanced by an intron sequence. Following transformation, both pCBJE1-14 and pCIBJES14 vectors resulted in cells expressing levels of JEV proteins similar to that observed with the pCDJE2-7 vector (Table 1). These results indicated that expression of the JEV proteins was influenced only by the transcriptional regulatory elements encoded in the recombinant plasmid. Neither the SV40 ori nor the intron sequence enhanced JEV protein expression in the cells used.

Epitope mapping of E protein expressed by a stably transformed cell line constitutively expressing JEV-specific gene products. Authenticity of the JEV E protein expressed by the JE-4B clone was demonstrated by epitope mapping by IFA using a panel of JEV E-specific murine MAbs. JEV HIAF and one irrelevant mouse ascitic fluid were used as positive and negative antibody controls, respectively. Four JEV-specific, six flavivirus subgroup-specific, and two flavivirus group-reactive MAbs reacted similarly with the 4B clone and with JEV-infected COS-1 cells (Table 2).

Detection of JEV E protein secreted by the JE-4B COS-1 cell line. An antigen capture ELISA, employing flavivirus group-reactive, anti-E MAbs 4G2 and 6B6C-1, was used to detect JEV E proteins that were secreted into the culture fluid by the COS-1 cell clone JE-4B. Antigen could be detected in the culture fluid the first day following seeding of the cells with maximum ELISA titers that ranged from 1:16 to 1:32.

Comparison of immune responses in mice vaccinated with pCDJE2-7 genetic vaccine and JEVAX. Plasmid pCDJE2-7 was used as a nucleic acid vaccine to induce an antibody response in mice by immunizing groups of five 3-week-old female ICR outbred mice. Mice were bled at 3, 6, 9, 23, 40, and 60 weeks after immunization, and antibody titers were determined by ELISA or by PRNT. As expected, sera from animals in the pCDNA3/CAT control group did not contain JEV antibody. All animals immunized with pCDJE2-7 and JEVAX seroconverted by 3 weeks after the first vaccination (Table 3). The antibody titers were similar irrespective of the number of doses

TABLE 2. Epitope mapping of E protein expressed by JE-4B, a pCDJE2-7 stably transformed clone of COS-1 cells, with JEV-reactive antibodies[a]

MAb or antiserum	Biological activity of MAb		IFA intensity of cells	
	Specificity	Biological function	JEV infected	4B
MAbs				
MC3	JEV specific		2+	2+
2F2	JEV specific	HI, N	4+	4+
112	JEV specific		4+	4+
503	JEV specific	N	4+	3+
109	Subgroup	HI	2+	1+
N.04	Subgroup	HI, N	3+	4+
201	Subgroup		1+	1+
203	Subgroup		4+	3+
204	Subgroup		2+	2+
301	Subgroup	HI	2+	2+
504	Flavivirus		4+	4+
6B6C-1	Flavivirus		2+	2+
3B4C-4	VEE		−	−
HIAF				
Anti-JEV			4+	3+
Anti-WEE			−	−
PBS			−	−

[a] VEE, Venezuelan equine encephalomyelitis virus; WEE, Western equine encephalomyelitis virus. −, negative.

TABLE 3. Persistence of the immune response in mice (five per group) immunized with pCDJE2-7 or JEVAX

Inoculation[a]	ELISA titer (log_{10})						$PRNT_{90}$ titer		
	3[b]	6	9	23	40	60[c]	3	6	9
pCDJE2-7									
1 dose	2.6–3.2	3.8–5.0	3.8–4.4	>3.2	>3.2	2.4, 2.4, 3.8, 4.4	<20	20	40–160
2 doses	2.6–3.8	4.4	3.8–4.4	>3.2	>3.2	2.6, 3.8, 3.8	<20	20–40	40–160
JEVAX, 2 doses	2.6–3.8	4.4–5.0	3.8–5.6	>3.2	>3.2	<2, <2, <2, 4.4	<20	20–40	20–160
pCDNA3/CAT, 2 doses	<100	<100	<100	ND[d]	ND	ND	<20	<20	<20

[a] Three-week-old mice were inoculated i.m. with one or two 100-μg doses of plasmid DNA or twice s.c. with one-fifth of the human dose of JEVAX.
[b] Weeks postimmunization.
[c] Individual serum titers.
[d] ND, not determined.

of pCDJE2-7 or JEVAX given. Mouse serum samples collected 9 weeks after immunization were also tested by Western blotting using purified JEV. Serum specimens from DNA-vaccinated mice, which had reactivity similar to that of JEV HIAF, detected E and prM proteins (Fig. 3). However, mouse serum from JEVAX-immunized mice reacted only with E protein. Comparable ELISA antibody titers were maintained in DNA-vaccinated groups for up to 60 weeks, at which time the experiment was terminated. Only one of four mice in the JEVAX group remained JEV antibody positive at 60 weeks postinoculation. These results demonstrated that one dose of JEV-specific nucleic acid vaccine was more effective in maintaining JEV antibody levels in mice than the commercially available vaccine JEVAX.

Comparison of various nucleic acid vaccine constructs and JEVAX for ability to induce JEV-reactive antibody in different age groups of mice. Similar amounts of JEV protein were expressed by COS-1 cells transformed by either pCDJE2-7, pCBJE1-14, or pCIBJES14. JEV antibody induction by these nucleic acid constructs was compared to results for JEVAX in two different age groups of mice. Three-day-old mixed-sex or 3-week-old female ICR outbred mice, 10 per group, were vaccinated i.m. with 50 or 100 μg of plasmid DNA or s.c. with 1/10 or 1/5 of the adult human dose of JEVAX, respectively. Serum specimens were collected at 7 weeks after immunization and tested at 1:400 or 1:1,600 by ELISA. Ninety to 100% of all 3-week-old mice that received pCBJE1-14, pCDJE2-7, pCIBJES14, or JEVAX had antibody titers of ≥1:1,600. However, a significant difference in antibody response was observed in 3-day-old groups that received various vaccines. None of the 3-day-old JEVAX-vaccinated mice had antibody titers higher than 1:400. All 3-day-old mice vaccinated with pCBJE1-14 had antibody titers higher than 1:1,600. Seroconversion of 100% was observed at 1:400 in 3-day-old mice that received pCDJE2-7 or pCIBJES14, but only 60% of both mouse groups were positive at 1:1,600. pCBJE1-14 was the most effective of three DNA constructs tested. The minimum dose of this DNA construct capable of providing 100% seroconversion (1:400 by ELISA) by i.m. immunization in 3-week-old mice was determined to be 25 μg (data not shown).

Protective immunity conferred by the nucleic acid vaccine. Mice immunized at 3 days of age were challenged by the i.p. route at 7 weeks postvaccination with the SA14 strain of JEV (50,000 PFU/100 μl) and observed for 3 weeks. One hundred percent of the animals that received various nucleic acid vaccine constructs were protected. In contrast, only 40 and 30% of mice that received JEVAX and pCDNA3/CAT, respectively, survived virus challenge (Fig. 4). These results suggested that the DNA vaccine could be effective as a neonatal vaccine. In contrast, JEVAX was not as effective in neonatal animals.

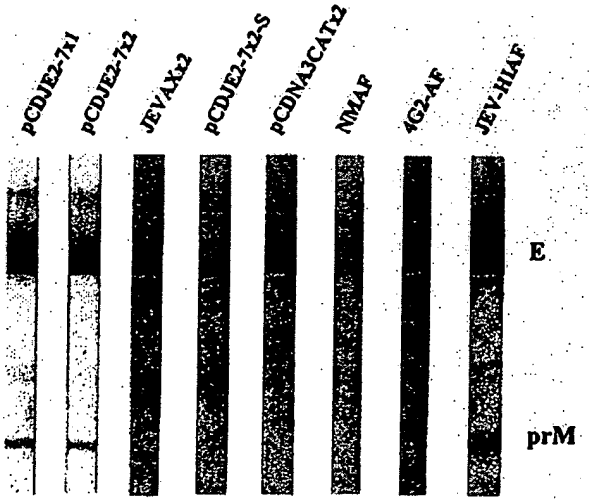


FIG. 3. JEV-specific reactivity of prechallenge and postchallenge serum samples obtained from mice immunized with DNA vaccine or JEVAX. Serum specimens collected from the mice used in the experiments represented in Tables 3 and 4 were randomly selected and tested at 1:1,000 dilution by Western blot analysis using purified JEV as the antigen. pCDJE2-7x2-S was the serum from one of the mice challenged at 4 days of age (Table 4). NMAF, 4G2-AF, and JEV HIAF were the mouse ascitic fluids included as normal mouse, E-specific, and JEV hyperimmune controls, respectively.

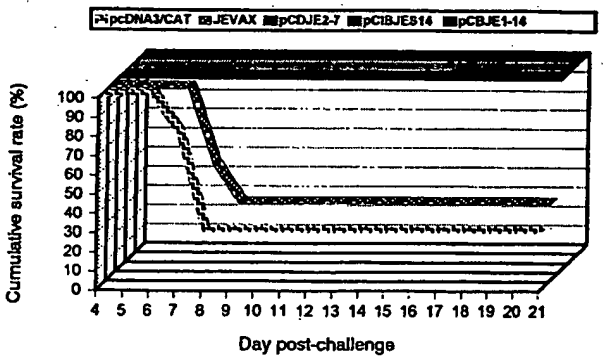


FIG. 4. Postchallenge survival rates of mice (10 per group) that were immunized with pCDJE2-7, pCBJE1-14, pCIBJES14, pcDNA3/CAT, or JEVAX at 3 days of age and challenged i.p. with 50,000 PFU of JEV (SA14) 7 weeks postimmunization. A P value of 0.003 was obtained by Fisher's exact test when the survival rate of the JEV DNA-immunized groups was compared with that of the pcDNA3/CAT or JEVAX group.

TABLE 4. Ability of maternal antibody from JEV nucleic acid-vaccinated female mice to protect their pups from fatal JE

Vaccinated mother[a]		JEV-challenged pups			
Vaccine	$PRNT_{90}$	Age (days)	No. of survivors/ total in litter	Avg survival time (days)	ELISA[b]
1 × pCDJE2-7	40	4	0/11	5.27	
2 × pCDJE2-7	80	4	12/12	NA[c]	12/12
2 × JEVAX	20	3	0/16	4.75	
2 × pCDNA3/CAT	<10	5	0/14	4.00	
1 × pCDJE2-7	20	15	5/11	10.0	5/5
2 × pCDJE2-7	40	14	8/12	13.75	7/8
2 × JEVAX	80	13	5/5	NA	5/5
2 × pCDNA3/CAT	<10	14	0/14	6.14	

[a] Mice were inoculated i.m. with one or two 100-μg doses of pCDJE2-7 DNA or twice s.c. with one-fifth of the adult human dose of JEVAX. Serum samples were collected 9 weeks postvaccination for PRNT testing prior to mating with nonimmune male.

[b] Number of JEV ELISA antibody-positive animals (titer ≥ 1:400)/number of survivors. Serum specimens were collected for testing 12 weeks after challenge.

[c] NA, not applicable.

Passive protection of neonatal mice correlated with the maternal antibody titer. Female 3-week-old ICR mice were vaccinated with one or two doses of pCDJE2-7 plasmid DNA (100 μg/100 μl) or twice with one-fifth of the adult human dose of JEVAX. For evaluation of passive protection by maternal antibody, pups were obtained from matings of experimental females with nonimmunized male mice. Pups were challenged by the i.p. route at 3 to 5 or 13 to 15 days after birth with SA14 virus (5,000 PFU/100 μl). Survival rates and average survival time correlated with the maternal neutralizing antibody titers (Table 4). One hundred percent of pups nursed by mothers with a PRNT of 1:80 survived viral infection regardless of the type of vaccine received by the mothers. None of the pups from mothers which received pCDNA3/CAT plasmid DNA survived (Table 4). Partial protection (45% [5 of 11 pups] to 67% [8 of 12 pups]) was observed in older pups that were nursed by the mothers which had serum PRNT titers of 1:20 and 1:40, respectively. However, none of the 3-day-old pups survived virus challenge when the mothers had a serum PRNT titer of 1:20 or 1:40. Maternally transferred antibody can only be detected in the circulation of the young mouse up to 40 days after birth. An appreciable level of maternally derived antibody is maintained in the circulation of the young mouse 24 days or more postpartum (1). JEV ELISA antibody detected in the serum of 97% (29 of 30) of the postchallenge pups at 12 weeks after virus challenge was unlikely to be residual maternally transferred antibody. The presence of JEV antibody in the surviving pups challenged at 3 to 4 or 13 to 15 days of age strongly suggested that maternal antibody did not provide sterilizing immunity to the pups. It also indicated that 3- to 4- or 13- to 15-day-old mice could mount an immune reaction to a live-virus challenge. Partial protection in older pups could be explained by the opportunity to accumulate a large quantity of passive antibody due to the length of nursing time before challenge. One randomly selected postchallenge serum sample also reacted with prM and E proteins by Western blotting (Fig. 3).

DISCUSSION

The flavivirus virion contains a capsid protein (C), a membrane protein (M), and an E protein. The prM MAbs, exhibiting weak or undetectable neutralizing activity in vitro, can provide passive protection following DEN2 virus challenge (16). However, the E protein plays a dominant role in generating neutralizing antibodies and providing protective immunity in the host. Passive transfer of JEV E-specific neutralizing MAbs has been shown to protect recipients from JEV-induced fatal encephalitis (3, 16, 32, 55). Antigenic and structural analysis using various panels of MAbs has shown that most of the E protein epitopes that elicit virus-neutralizing antibodies are conformationally dependent (9, 40). Coexpression of both proteins as type I transmembrane proteins is essential to maintain proper E conformation and prevent the E protein from undergoing irreversible, low-pH-catalyzed conformational changes (8–10, 19, 50). A 2-kb genomic region, from the internal signal peptide at the carboxyl terminus of C to the transmembrane domain at the carboxyl terminus of the E gene, is essential for expressing authentic proteins. These authentic prM and E proteins are able to self-assemble into virus-like particles in cells infected by either recombinant vaccinia virus or alphavirus vector or in cells transformed by recombinant plasmid DNA (4, 19, 22, 48; Hunt and Chang, unpublished data).

A gene cassette including the elements listed above was amplified from SA14 virus by RT-PCR in the present study. Optimal sequence composition surrounding the translation initiation site (−9 to +4) was incorporated into the 14DV398 amplifying primer (2, 26, 27) (Fig. 1). Recombinant plasmids containing the CMV early gene promoter/enhancer and the BHG poly(A) terminator as transcription regulatory elements expressed JEV proteins with the highest efficiency in three different cell lines. Protein expression and the serological response of mice immunized with DNA vaccine were not influenced by the presence or absence of the SV40 ori or an intron sequence in recombinant plasmids. Virus-specific proteins, secreted into culture medium, could be detected by antigen capture ELISA as early as 48 h after plasmid transformation (data not shown). The authenticity of the E protein produced by the pCDJE2-7 stably transformed cell line, JE-4B, was demonstrated by MAb epitope mapping.

Vaccine potential and characteristics of various eukaryotic plasmids that express flavivirus prM and E proteins are summarized in Tables 5 and 6. All constructs listed had the same transcriptional control elements and similar viral gene cassettes. DEN2 plasmid, which contains prM and 91% of E, is the only exception (Table 6). The JEV DNA vaccine reported in this study is the only construct that stimulated complete protective immunity in mice by a single dose of vaccine given by the i.m. route (Table 5). Sequences surrounding the translation initiation site and the composition of the signal peptide preceding the prM protein are the two major differences among the constructs that may contribute to increasing the vaccine potential of our construct (Table 6). Conserved features of the sequences which flank vertebrate translation initiation sites include a strong preference for purine at the −3 position; a higher frequency of G at positions −9, −6, −3, and +4; and a preference for A or C at positions −5, −4, −2, and −1 (2). Instead of the sequence used in previous publications, the sequence used in our construct was −9 · GCCGCCGCC ATGG, which fits the general criteria listed above. Although less than 1% of eukaryotic mRNA sequences exhibit this sequence, the experimental data have suggested that this sequence provides exceptionally high levels of translation potential (2, 26).

Signal peptides determine translocation and orientation of inserted protein, hence the topology of prM and E. Signal peptide differences in our plasmid construct may account for the efficient translocation and correct topology, thus increasing prM and E secretion. A machine-learning program using neu-

TABLE 5. Vaccine potential of various eukaryotic plasmids that express flavivirus prM and E proteins[a]

Virus	In vitro secretion of EPs	Immunization			Protection from virus challenge	Reference
		Dosage	Route/method	Neutralizing antibody[b]		
JE	Yes	25–100 μg × 1	i.m./needle	Yes ($1:20–1:160_{90\%}$)	100%	This report
	ND	100 μg × 2	i.m./needle	No	Partial	30
	ND	10–100 μg × 2	i.m. or i.d./needle	Yes ($1:10–1:20_{90\%}$)	100%	24
MVE	Yes	100 μg × 4	i.m./needle	ND	Partial	4
	Yes	1–2 μg × 2–4	i.d./gene gun	Yes ($80–320_{50\%}$)	100%	4
SLE	ND	100 μg × 2	i.m./needle	No	Partial	38
CEE	ND	1 μg × 1–2	i.d./gene gun	Yes ($1:100–1:1,600_{80\%}$)	100%	49
RSSE	ND	1 μg × 1–2	i.d./gene gun	ND	100%	49
DEN2	ND	200 μg × 3	i.d./needle	Yes ($1:10–1:320_{50\%}$)	None	17

[a] MVE, Murray Valley encephalitis; CEE, Central European encephalitis; RSSE, Russian spring-summer encephalitis; i.d., intradermal; ND, not done.
[b] Plaque reduction neutralization titer followed by percentage reduction endpoint used in the test.

ral networks trained on eukaryotes (SignalP-NN at http://www.cbs.dtu.dk/services/) was applied to test the efficiency of the prM signal peptide sequence in the different plasmid constructs (34) (Table 6). The most probable location and orientation of transmembrane helices in the prM-E protein were then determined by a hidden Markov model-trained computer program (6 [TMHMM at http://www.cbs.dtu.dk/services/]). SignalP-NN searches correctly predicted the signal peptidase cleavage site of all constructs. However, a considerable difference in cleavage potential (C score, between 0.578 and 1.000) was observed (Table 6). Cleavage potential differences may be influenced by the amino acid composition and length of the h region in various constructs (44).

The TMHMM program correctly predicted five transmembrane helices encoded in the prM-E protein. Significant difference in the probable orientation of the first transmembrane helix was observed in three JEV constructs (Fig. 5). In our pCDJE2-7 construct, the first 12 amino acids of the n region form a short loop in the cytoplasmic side that causes the following h region (transmembrane helix) to be inserted in a tail orientation. Secretion of JEV protein could be detected by antigen capture ELISA in pCDJE2-7 transient expression studies in which less than 5% of the cells were positive by IFA (data not shown). Thus, there is a high probability that prM and E proteins expressed by pCDJE2-7 would be expressed in the correct orientation, as type I transmembrane proteins (Fig. 5A). There is also a high probability that the prM protein of pcDNA3JEME could be expressed as a type II membrane protein with its transmembrane h region inserted in a head orientation because of the absence of positively charged amino acids in its n region (Fig. 5B). Efficient protein synthesis in conjunction with correct topology of expressed prM and E (Fig. 5A) would most likely enhance EP formation and secretion in transformed cells.

Another characteristic that could explain the excellent vaccine potential of our JEV construct is its ability to produce EPs which have a virus-like polymeric structure that enhances antigenic stability and provides a high-density presentation to antigen-presenting cells, such as macrophages, dendritic cells, and Langerhans cells (5). When DNA is given by the i.m. route, the majority of antigen is expressed by non-antigen-presenting muscle cells. The efficacy of a DNA vaccine is therefore dependent on transfection of antigen-presenting cells or to reprocessing of antigen derived from other cells. Muscle cells transfected by our construct could conceivably synthesize and secrete EPs, which are highly immunogenic and have been shown to elicit good cellular and humoral responses (22, 23).

Genetic JEV vaccine that induced a completely protective immunity in neonatal mice and a maternally transferable protective immunity in young adult mice by a single i.m. immunization was demonstrated in this study. Additional studies are planned to address the effectiveness of a DNA vaccine in overcoming the potential influence of maternally transferred flavivirus antibodies on the induction of JEV antibody in neonatal mice.

Immunization of pigs is a theoretical means of interrupting transmission and amplification of JEV and thereby preventing human infections (43). The JEV DNA vaccine could also be used as a veterinary vaccine in pregnant sows to prevent JEV-induced stillbirth and abortion (51, 53). Maternally transferred antibody could also interrupt piglets as the JEV-amplifying host and thus reduce human infection.

TABLE 6. Characteristics of various eukaryotic plasmids expressing flavivirus prM and E proteins

Virus[a]	Plasmid	Sequence surrounding translation initiation site	Amino acids preceding prM protein[b]	SP potential (C score)[c]	Reference
JE	pCDJE2-7	−9•GCCGCCGCCATGG•+4	MGRKQNKRGGNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	This report
	pJME	−9•GGCTCAATCATGG•+4	MWLASLAVVIACAGA /MKL	Yes (0.578)	30
	pCDNA3JEME	−9•GAATTCACCATGG•+4	MNEGSIMWLASLAVVIACAGA /MKL	Yes (0.921)	24
MVE	pCDNA3.prM-E	−9•TGATTTCAAATGT•+4	MSKKRGGSETSVLMVIFMLIGFAAA /LKL	Yes (0.819)	4
SLE	pSLE1	?	?LDTINRRPSKKRGGTRSLLGLAALIGLASS /LQL	Yes (0.709)	38
DEN2	p1012D2ME	?	?AGMIIMLIPTVMA /FHL	Yes (0.646)	17
TBE	SV-PE_{wt}	−9•GCGGCCGCCATGG•+4	MVGLQKRGKRRSATDWMSWLLVITLLGMTLA /ATV	Yes (1.000)	48
RSSE	pWRG7077	−9•GTAGACAGGATGG•+4	MGWLLVVVLLGVTLA /ATV	Yes (0.762)	50
CEE	pWRG7077	−9•ACGGACAGGATGG•+4	MSWLLVITLLGMTIA /ATV	Yes (0.609)	50

[a] Abbreviations are as given in Table 5, footnote *a*.
[b] Single amino acid code. Positively charged amino acid is indicated by bold letter. Signal peptidase cleavage site is indicated by /.
[c] Cleavage potential of signal peptide (SP) predicted by SignalP-NN at http://www.cbs.dtu.dk/services (34).

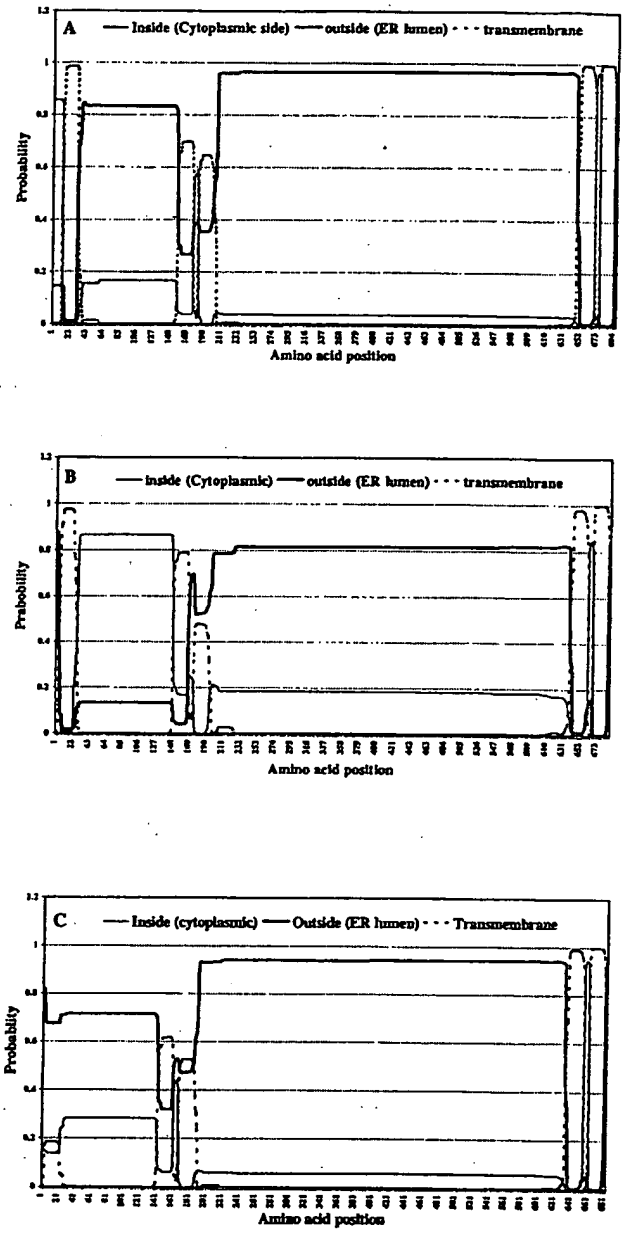


FIG. 5. Graphic representation, generated by the TMHMM program, indicating probable orientations of five transmembrane helices in the prM-E protein expressed by pCDJE2-7 (A), pcDNA3JEME (B), and pJME (C). ER, endoplasmic reticulum.

ACKNOWLEDGMENTS

We thank K. Yasui and M.-J. Zhang for providing JEV MAbs and J. Roehrig and B. Miller for useful discussion and advice. We thank D. Holmes, C. Lin, N. Frank, and T. Springfield for superb technical assistance and animal care.

REFERENCES

1. **Appleby, P., and D. Catty.** 1983. Transmission of immunoglobulin to foetal and neonatal mice. J. Reprod. Immunol. 5:203–213.
2. **Cavener, D. R., and S. C. Ray.** 1991. Eukaryotic start and stop translation sites. Nucleic Acids Res. 19:3185–3192.
3. **Cecilia, D., D. A. Gadkari, N. Kedarnath, and S. N. Ghosh.** 1988. Epitope mapping of Japanese encephalitis virus envelope protein using monoclonal antibodies against an Indian strain. J. Gen. Virol. 69:2741–2747.
4. **Colombage, G., R. Hall, M. Pavy, and M. Lobigs.** 1998. DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. Virology 250:151–163.
5. **Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo, Jr.** 1996. DNA-based immunization by in vivo transfection of dendritic cells. Nat. Med. 2:1122–1128.
6. **Erik, L. L., S. G. von Heijne, and A. Krogh.** 1998. A hidden Markov model for predicting transmembrane helices in protein sequences, p. 175–182. *In* J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff, and C. Sensen (ed.), Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology. AAAI Press, Menlo Park, Calif.
7. **Gentry, M. K., E. A. Henchal, J. M. McCown, W. E. Brandt, and J. M. Dalrymple.** 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. Am. J. Trop. Med. Hyg. 31:548–555.
8. **Guirakhoo, F., R. A. Bolin, and J. T. Roehrig.** 1992. The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein. Virology 191:921–931.
9. **Heinz, F. X., R. Berger, W. Tuma, and C. Kunz.** 1983. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology 126:525–537.
10. **Heinz, F. X., K. Stiasny, G. Puschner-Auer, H. Holzmann, S. L. Allison, C. W. Mandl, and C. Kunz.** 1994. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein E by the heterodimeric association with protein prM. Virology 198:109–117.
11. **Hennessy, S., Z. Liu, T. F. Tsai, B. L. Strom, C. M. Wan, H. L. Liu, T. X. Wu, H. J. Yu, Q. M. Liu, N. Karabatsos, W. B. Bilker, and S. B. Halstead.** 1996. Effectiveness of live-attenuated Japanese encephalitis vaccine (SA14-14-2): a case-control study. Lancet 347:1583–1586.
12. **Hunt, A. R., and C. H. Calisher.** 1979. Relationships of bunyamwera group viruses by neutralization. Am. J. Trop. Med. Hyg. 28:740–749.
13. **Igarashi, A.** 1978. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and Chikungunya viruses. J. Gen. Virol. 40:531–544.
14. **Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels.** 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am. J. Trop. Med. Hyg. 41:576–580.
15. **Kimura-Kuroda, J., and K. Yasui.** 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of Japanese encephalitis virus, using monoclonal antibodies. J. Virol. 45:124–132.
16. **Kimura-Kuroda, J., and K. Yasui.** 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. J. Immunol. 141:3606–3610.
17. **Kochel, T., S. J. Wu, K. Raviprakash, P. Hobart, S. Hoffman, K. Porter, and C. Hayes.** 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice. Vaccine 15:547–552.
18. **Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, N. Kanesa-Thasan, J. J. Smucny, C. H. Hoke, Jr., and F. A. Ennis.** 1998. Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Vaccine 16:842–849.
19. **Konishi, E., and P. W. Mason.** 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the premembrane protein. J. Virol. 67:1672–1675.
20. **Konishi, E., S. Pincus, B. A. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason.** 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. Virology 185:401–410.
21. **Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason.** 1992. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. Virology 190:454–458.
22. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology 188:714–720.
23. **Konishi, E., K. S. Win, I. Kurane, P. W. Mason, R. E. Shope, and F. A. Ennis.** 1997. Particulate vaccine candidate for Japanese encephalitis induces long-lasting virus-specific memory T lymphocytes in mice. Vaccine 15:281–286.
24. **Konishi, E., M. Yamaoka, Khin-Sane-Win, I. Kurane, and P. W. Mason.** 1998. Induction of protective immunity against Japanese encephalitis in mice by immunization with a plasmid encoding Japanese encephalitis virus premembrane and envelope genes. J. Virol. 72:4925–4930.
25. **Kozak, M.** 1984. Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. Nucleic Acids Res. 12:857–872.
26. **Kozak, M.** 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. 196:947–950.
27. **Kozak, M.** 1997. Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. EMBO J. 16:2482–2492.
28. **Ku, C. C., C. C. King, C. Y. Lin, H. C. Hsu, L. Y. Chen, Y. Y. Yueh, and G. J. Chang.** 1994. Homologous and heterologous neutralization antibody re-

sponses after immunization with Japanese encephalitis vaccine among Taiwan children. J. Med. Virol. 44:122–131.
29. Kuno, G., G. J. Chang, K. R. Tsuchiya, N. Karabatsos, and C. B. Cropp. 1998. Phylogeny of the genus *Flavivirus*. J. Virol. 72:73–83.
30. Lin, Y. L., L. K. Chen, C. L. Liao, C. T. Yeh, S. H. Ma, J. L. Chen, Y. L. Huang, S. S. Chen, and H. Y. Chiang. 1998. DNA immunization with Japanese encephalitis virus nonstructural protein NS1 elicits protective immunity in mice. J. Virol. 72:191–200.
31. Liu, Z. L., S. Hennessy, B. L. Strom, T. F. Tsai, C. M. Wan, S. C. Tang, C. F. Xiang, W. B. Bilker, X. P. Pan, Y. J. Yao, Z. W. Xu, and S. B. Halstead. 1997. Short-term safety of live attenuated Japanese encephalitis vaccine (SA14-14-2): results of a randomized trial with 26,239 subjects. J. Infect. Dis. 176:1366–1369.
32. Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason. 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J. Gen. Virol. 70:2037–2049.
33. McCown, J., M. Cochran, R. Putnak, R. Feighny, J. Burrous, E. Henchal, and C. Hoke. 1990. Protection of mice against lethal Japanese encephalitis with a recombinant baculovirus vaccine. Am. J. Trop. Med. Hyg. 42:491–499.
34. Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Int. J. Neural Syst. 8:581–599.
35. Nitayaphan, S., J. A. Grant, G. J. Chang, and D. W. Trent. 1990. Nucleotide sequence of the virulent SA-14 strain of Japanese encephalitis virus and its attenuated vaccine derivative, SA-14-14-2. Virology 177:541–552.
36. Nothdurft, H. D., T. Jelinek, A. Marschang, H. Maiwald, A. Kapaun, and T. Loscher. 1996. Adverse reactions to Japanese encephalitis vaccine in travellers. J. Infect. 32:119–122.
37. Obijeski, J. F., D. H. Bishop, E. L. Palmer, and F. A. Murphy. 1976. Segmented genome and nucleocapsid of La Crosse virus. J. Virol. 20:664–675.
38. Phillpotts, R. J., K. Venugopal, and T. Brooks. 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Arch. Virol. 141:743–749.
39. Poland, J. D., C. B. Cropp, R. B. Craven, and T. P. Monath. 1990. Evaluation of the potency and safety of inactivated Japanese encephalitis vaccine in US inhabitants. J. Infect. Dis. 161:878–882.
40. Roehrig, J. T., R. A. Bolin, and R. G. Kelly. 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology 246:317–328.
41. Roehrig, J. T., A. R. Hunt, A. J. Johnson, and R. A. Hawkes. 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology 171:49–60.
42. Roehrig, J. T., J. H. Mathews, and D. W. Trent. 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology 128:118–126.
43. Rosen, L. 1986. The natural history of Japanese encephalitis virus. Annu. Rev. Microbiol. 40:395–414.
44. Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara, and T. Omura. 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc. Natl. Acad. Sci. USA 89:16–19.
45. Sakaguchi, M., M. Yoshida, W. Kuroda, O. Harayama, Y. Matsunaga, and S. Inouye. 1997. Systemic immediate-type reactions to gelatin included in Japanese encephalitis vaccines. Vaccine 15:121–122.
46. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. Sato, T., C. Takamura, A. Yasuda, M. Miyamoto, K. Kamogawa, and K. Yasui. 1993. High-level expression of the Japanese encephalitis virus E protein by recombinant vaccinia virus and enhancement of its extracellular release by the NS3 gene product. Virology 192:483–490.
48. Schalich, J., S. L. Allison, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz. 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. J. Virol. 70:4549–4557.
49. Schmaljohn, C., L. Vanderzanden, M. Bray, D. Custer, B. Meyer, D. Li, C. Rossi, D. Fuller, J. Fuller, J. Haynes, and J. Huggins. 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. 71:9563–9569.
50. Stocks, C. E., and M. Lobigs. 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. 72:2141–2149.
51. Takashima, I., T. Watanabe, N. Ouchi, and N. Hashimoto. 1988. Ecological studies of Japanese encephalitis virus in Hokkaido: interepidemic outbreaks of swine abortion and evidence for the virus to overwinter locally. Am. J. Trop. Med. Hyg. 38:420–427.
52. Tsai, T. F., G. J. Chang, and Y. X. Yu. 1999. Japanese encephalitis vaccines, p. 672–710. *In* A. P. Stanley and W. A. Orenstein (ed.), Vaccines, 3rd ed. W. B. Saunders, Philadelphia, Pa.
53. Ueba, N., T. Kimura, S. Nakajima, T. Kurimura, and T. Kitaura. 1978. Field experiments on live attenuated Japanese encephalitis virus vaccine for swine. Biken J. 21:95–103.
54. Yasuda, A., J. Kimura-Kuroda, M. Ogimoto, M. Miyamoto, T. Sata, T. Sato, C. Takamura, T. Kurata, A. Kojima, and K. Yasui. 1990. Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express PreM and E glycoproteins of Japanese encephalitis virus. J. Virol. 64:2788–2795.
55. Zhang, M. J., M. J. Wang, S. Z. Jiang, and W. Y. Ma. 1989. Passive protection of mice, goats, and monkeys against Japanese encephalitis with monoclonal antibodies. J. Med. Virol. 29:133–138.

JOURNAL OF VIROLOGY, Sept. 2000, p. 8213–8217
0022-538X/00/$04.00+0

Vol. 74, No. 17

Sex Differences in Seoul Virus Infection Are Not Related to Adult Sex Steroid Concentrations in Norway Rats

SABRA L. KLEIN, BRIAN H. BIRD, AND GREGORY E. GLASS*

Department of Molecular Microbiology and Immunology, The Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205-2179

Received 14 April 2000/Accepted 8 June 2000

Field studies of hantavirus infection in rodents report that a higher percentage of infected individuals are males than females. To determine whether males were more susceptible to hantavirus infection than females, adult male and female Long Evans rats (*Rattus norvegicus*) were inoculated with doses of Seoul virus ranging from 10^{-4} to 10^{6} PFU. The 50% infective doses (ID_{50}) were not significantly different for male and female rats ($10^{0.05}$ and $10^{0.8}$ PFU, respectively). To determine whether sex differences in response to infection were related to circulating sex steroid hormones, sex steroid concentrations were manipulated and antibody responses and virus shedding were assessed following inoculation with the ID_{90}. Regardless of hormone treatment, males had higher anti-Seoul virus immunoglobulin G (IgG) and IgG2a (i.e., Th1) responses than females and IgG1 (i.e., Th2) responses similar to those of females. Males also shed virus in saliva and feces longer than females. Manipulation of sex steroids in adulthood did not alter immune responses or virus shedding, suggesting that sex steroids may organize adult responses to hantavirus earlier during ontogeny.

Hantaviruses are negative-sense RNA viruses (family *Bunyaviridae*) encompassing over 20 different viruses that are each carried by a different host species, with rodents serving as the primary reservoirs (18). Field surveys of several rodent species, including brush mice, deer mice, harvest mice, bank voles, and cotton rats, indicate that males are more commonly infected than females (4, 8, 11, 19, 20, 27). Because these studies used serology to determine hantavirus infection, sex differences in infection could reflect either a lack of infection or the absence of sustained antibody production in females. Experimental inoculation of female rodents with hantavirus, however, illustrates that females produce long-lasting, detectable antibody (22). Alternatively, sex differences in hantavirus prevalence may reflect differences in endocrine-immune interactions (15). The extent to which sex steroids affect immune responses against hantavirus infection has not been examined.

In contrast to other rodent species, sex differences in hantavirus prevalence have not been reported consistently among natural populations of Norway rats. Among adult rats, however, males (90%) tend to be infected with Seoul virus more often than females (75%) (7, 10). Seoul virus is hypothesized to be transmitted via wounding, and adult male rats are more likely to be wounded than either females or juvenile males (10). Thus, sex differences in hantavirus prevalence may reflect complex interactions between behavior and physiology. The first goal of this study was to control for sex differences in exposure and determine whether males were more susceptible to hantavirus infection than females. At 70 to 80 days of age, 5 to 10 male and 5 to 10 female Long Evans rats (*Rattus norvegicus*) were inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (with Earle's salts; Meditach Cellgro, Va.). Seoul virus was obtained from the U.S. Army Medical Research Institute of Infectious Diseases (Ft. Detrick, Md.), where the virus was isolated from neonatal rat brains and passaged four times in Vero E6 cells. Blood samples were obtained from each animal prior to infection and then 10, 20, 30, and 40 days postinoculation under anesthesia with methoxyflurane vapors (Metofane; Schering Plough, Union, N.J.).

Plasma was used to detect anti-Seoul virus immunoglobulin G (IgG) using an enzyme-linked immunosorbent assay in which microtiter plates were coated overnight at 4°C with gamma-irradiated Vero E6 cells infected with Seoul virus or gamma-irradiated uninfected Vero E6 cells diluted 1:500 in carbonate buffer. Thawed plasma samples, as well as positive control samples (i.e., pooled plasma from rats previously determined to have anti-Seoul virus IgG) and negative control samples (i.e., pooled plasma from Seoul virus-naive rats), were diluted 1:100 in phosphate-buffered saline (PBS)–Tween (PBS-T) with 2% fetal bovine serum and added in duplicate to antigen-coated wells containing either infected or uninfected Vero E6 cells. The plates were sealed, incubated at 37°C for 1 h, and washed with PBS-T, and secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, Md.; alkaline phosphatase-conjugated anti-rat IgG [heavy plus light chains], horseradish peroxidase-conjugated anti-rat IgG1, or horseradish peroxidase-conjugated anti-rat IgG2a diluted 1:400 in PBS with 2% fetal bovine serum) was added. The plates were resealed, incubated for 1 h at 37°C, and washed with PBS-T, and substrate buffer (0.5 mg of *p*-nitrophenyl phosphate per ml diluted in diethanolamine substrate buffer for alkaline phosphatase reactions or tetramethylbenzidine for horseradish peroxidase reactions) was added to each well. Plates were protected from light during the enzyme-substrate reaction, which was terminated after 30 to 45 min by adding 1.5 M NaOH to each well for alkaline phosphatase reactions or 2 N H_2SO_4 to each well for horseradish peroxidase reactions. The optical density (OD) was measured at 405 nm for alkaline phosphatase reactions and 450 nm for horseradish peroxidase reactions, and the average OD for each set of uninfected Vero E6 duplicates was subtracted from the average OD for each set of infected Vero E6 duplicates. Samples were considered positive if the average adjusted OD was ≥0.100. To minimize intra- and interplate variability, the average adjusted OD for each sample

* Corresponding author. Mailing address: Department of Molecular Microbiology and Immunology, Johns Hopkins School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205-2179. Phone: (410) 955-3708. Fax: (410) 955-0105. E-mail: ggurrigl@jhsph.edu.

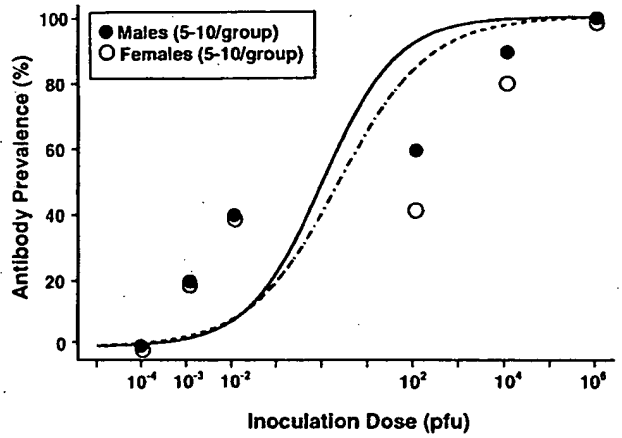


FIG. 1. Antibody prevalence among intact male and female rats inoculated with either 10^{-4}, 10^{-3}, 10^{-2}, 10^{2}, 10^{4}, or 10^{6} PFU of Seoul virus. Data are presented as percentages of individuals producing detectable antibody (i.e., adjusted average OD ≥ 0.100) against Seoul virus by day 40 postinoculation, with the fitted logistic regression curves for both males (solid line) and females (dashed line) included. Equal percentages of males and females seroconverted in response to each dose of Seoul virus ($P > 0.05$ in each case).

was expressed as a percentage of its plate-positive control OD for statistical analyses (9).

Antibody prevalence (i.e., the number of animals with detectable anti-Seoul virus IgG) by day 40 postinoculation was compared between males and females using chi-square analyses. Antibody prevalence was assessed 40 days after inoculation because previous studies illustrate that hantavirus-specific antibody is detectable 15 to 30 days postinoculation (7, 14, 22). Antibody prevalence did not differ between males and females at any of the six doses of Seoul virus ($P > 0.05$). Logistic regression was used to compare the infective-dose (ID) curves and estimate the 50% ID (ID_{50}). The ID_{50} did not differ significantly between males (mean ± standard deviation, 1.1 ± 2.0 PFU) and females (7.6 ± 2.0 PFU) (Fig. 1).

Although the prevalence of males and females that became infected did not differ, studies of other viral infections suggest that patterns of immune responses differ between the sexes and are mediated by sex steroid hormones (1, 15, 29). Thus, males and females may differ because testosterone suppresses and estradiol enhances several aspects of immune function (1, 15, 17, 24, 26, 29). The second aim of this study was to examine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection. Immunologically, patterns of helper T (Th) cell responses (i.e., Th1 or Th2) differ between males and females, with males exhibiting elevated Th1 responses (i.e., elevated gamma interferon, interleukin-2 [IL-2], and IgG2a levels) and females exhibiting increased Th2 responses (i.e., higher IL-4, IL-5, IL-6, and IL-10 levels) (5, 12, 13). Treatment of males with estradiol and females with testosterone prior to infection with pathogens, such as coxsackievirus, reverses the Th responses, suggesting that hormones can modify immune responses to virus infection (12, 13). To determine whether adult sex steroid hormone concentrations influence immune responses and virus shedding following hantavirus infection, at 70 to 80 days of age 20 male and 20 female rats were bilaterally gonadectomized under ketamine (80 mg/kg of body mass)–xylazine (6 mg/kg) anesthesia (Phoenix Pharmaceutical, St. Joseph, Mo.) and given 2 weeks to recover from surgery. After recovery, 10 castrated males were each subcutaneously implanted with a 30-mm Silastic capsule (inside diameter [i.d.] = 1.47 mm, outside diameter [o.d.] = 1.96 mm) containing 20 mm of testosterone propionate (Sigma, St. Louis, Mo.). The remaining 10 castrated males, as well as 10 intact males, were each implanted with an empty capsule of equal length. Ten ovariectomized females were each subcutaneously implanted with a 15-mm Silastic capsule (i.d. = 1.47 mm, o.d. = 1.96 mm) containing 10 mm of estradiol benzoate (Sigma). The remaining 10 ovariectomized females and 9 intact females were each implanted with an empty Silastic capsule of equal length. Silastic capsule length was based on previous reports that these hormone doses (i.e., the length of the Silastic capsule) are sufficient to maintain physiological testosterone and estradiol concentrations in male and female rats, respectively (25). At the time the Silastic capsules were implanted, all animals received an intraperitoneal inoculation of 10^4 PFU of Seoul virus (strain SR-11) suspended in 0.2 ml of Eagle minimum essential medium (i.e., the ID_{90} from the first experiment). Blood, saliva, and fecal samples were then obtained from each animal on days 0, 10, 15, 20, 30, and 40 postinoculation under anesthesia with methoxyflurane vapors. Saliva samples were collected from anesthetized rats after injecting them intraperitoneally with 2.5 mg of pilocarpine HCl (Sigma) per kg of body mass suspended in 0.9% sterile saline (6). After samples were collected on day 40 postinoculation, animals were killed and seminal vesicles were removed from the males and weighed as an index of long-term testosterone concentrations. All procedures described in this paper were approved by the Johns Hopkins Animal Care and Use Committee (protocol number RA98H536) and the Johns Hopkins Office of Health, Safety, and Environment (registration number A9902030102).

Relative seminal vesicle weights (i.e., corrected for body mass) were higher among intact males (0.282 ± 0.13 g) and castrated males treated with testosterone (0.326 ± 0.12 g) than among castrated males (0.095 ± 0.06 g) [$F(2, 29) = 12.75, P < 0.05$]. Plasma testosterone concentrations in males and estradiol concentrations in females were assayed by radioimmunoassay using the manufacturer's protocols (ICN Biochemicals, Inc., Carson, Calif.). Testosterone concentrations were higher for intact males and castrated males treated with testosterone than for castrated male rats; castrated males treated with testosterone also had higher testosterone concentrations than intact males on days 10, 15, 20, and 30, but not on day 40, postinoculation [$F(10, 179) = 19.30, P < 0.05$] (Table 1). Plasma estradiol concentrations were higher for intact females and ovariectomized females treated with estradiol than for ovariectomized females 10, 15, 20, 30, and 40 days postinoculation; ovariectomized females treated with estradiol also had higher estradiol concentrations than intact females on days 10, 15, 20, 30, and 40 postinoculation [$F(10, 173) = 10.29, P < 0.05$] (Table 1).

Manipulation of testosterone concentrations in males and estradiol concentrations in females did not affect production of antibody against Seoul virus ($P > 0.05$). Overall, males had higher anti-Seoul virus IgG responses than females on days 20, 30, and 40 postinoculation, regardless of hormone treatment [$F(5, 353) = 18.72, P < 0.05$] (Table 2). Male rats also had higher anti-Seoul virus IgG2a responses than females on days 30 and 40 postinoculation despite hormone manipulation [$F(5, 353) = 7.81, P < 0.05$] (Fig. 2A). In contrast, females tended to show higher IgG1 responses than males on days 30 and 40 postinoculation, though this did not reach statistical significance ($P > 0.05$) (Fig. 2B).

Viral RNA was identified using nested reverse transcription-PCR (RT-PCR), and the presence of virus in saliva and feces was used to determine whether virus was shed. Viral RNA was isolated using a guanidine isothiocyanate procedure (3). For

TABLE 1. Sex steroid hormone concentrations[a]

Hormone and group	Hormone concn (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Testosterone						
Intact males	0.69 ± 0.17*	0.84 ± 0.17*	1.13 ± 0.36*	0.92 ± 0.25*	0.77 ± 0.19*	0.70 ± 0.13*
Castrated males	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
T-treated males	0.00 ± 0.00	8.24 ± 0.74*†	6.28 ± 0.91*†	6.62 ± 1.18*†	2.73 ± 0.42*†	0.71 ± 0.28*
Estradiol						
Intact females	25.8 ± 6.81*	27.0 ± 5.57*	20.8 ± 8.39*	25.9 ± 7.78*	38.2 ± 10.1*	55.2 ± 10.2*
Ovx females	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
E_2-treated females	0.00 ± 0.00	166.6 ± 20.6*†	123.1 ± 21.9*†	87.5 ± 8.9*†	162.3 ± 18.8*†	109.7 ± 19.3*†

[a] Sex steroid hormone concentrations in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] Testosterone levels are in nanograms per milliliter, and estradiol levels are in picograms per milliliter. An asterisk indicates that intact and hormone-treated animals had higher hormone concentrations than their gonadectomized counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$). A dagger indicates that hormone-treated animals had higher sex steroid concentrations than their intact counterparts on the corresponding day, based on an analysis of variance ($P < 0.05$).

RNA isolation from saliva, samples were collected from each rat and added to Trizol LS reagent (Life Technologies, Rockville, Md.) at a 3:1 ratio, with RNase-free glycogen (10 μg) added as a carrier. For RNA isolation from feces, approximately 100 mg of feces was homogenized in Tris-EDTA buffer (pH 8.0) and centrifuged at 12,000 × *g* for 10 min at 4°C; supernatants were collected, incubated with proteinase K (50 μg/ml; Life Technologies) and 0.5% sodium dodecyl sulfate at 50°C for 30 min to digest proteins, and then added to Trizol LS at a 3:1 ratio. To separate, precipitate, and resuspend viral RNA, the manufacturer's protocol was used (Trizol LS; Life Technologies).

For RT-PCR, a 280-bp nucleotide sequence of the SR-11 small (S) genome was amplified using two 20-bp primers, HTN-S4 (5′ GATAGGTGTCCACCAACATG 3′) and HTN-S6 (5′ AGCTCTGGATCCATGTCATC 3′), that amplified positions 979 through 1259 (3). The DNA fragment obtained from the RT-PCR was further amplified using primers HTN-S3 (5′ GCCTTCTTTTCTATACTTCAGG 3′) and HTN-S5 (5′ CCAGGCAACCATAAACATAAC 3′), designed to amplify a 176-bp nucleotide sequence (positions 1031 through 1207). First-strand cDNA was prepared using the GeneAmp RNA PCR kit protocol (Perkin-Elmer, Branchburg, N.J.), incubated in a DNA thermocycler (Techne Genius) at 42°C for 15 min, 99°C for 5 min, and 5°C for 5 min, and then held at 4°C. The reaction mixture contained 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, 1 U of RNase inhibitor, and 2.5 U of murine leukemia virus reverse transcriptase. The positive control was SR-11 RNA isolated from virus stock, and the negative control was diethyl pyrocarbonate water that was included in the cDNA syntheses and primary and secondary amplifications.

The 280-bp sequence was amplified in a 100-μl reaction mixture containing 20 μl of the cDNA, 0.3 μM HTN-S6 primer, and 2.5 U of polymerase (AmpliTaq; Perkin-Elmer). Reactions were amplified for one cycle at 94°C for 3 min and 40 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, followed by 10 min at 72°C. The nested 176-bp sequence was amplified in a 100-μl reaction mixture containing 2 μl of the product of the first DNA amplification, 20 μM HTN-S3 primer, 20 μM HTN-S5 primer, 10 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 2.5 U of polymerase. Nested-PCR products were amplified using the same cycle series as was used for the primary amplification. The PCR products were electrophoresed on a 4% gel (3% NuSieve plus 1% SeaKem; FMC Bioproducts, Rockland, Maine), stained with ethidium bromide, and examined for bands of the appropriate size. Randomly selected positive PCR products from saliva and fecal samples from males and females, as well as positive and negative control products, were purified using QIAquick (Qiagen, Valencia, Calif.) and sequenced.

Virus shedding in saliva and feces was not altered by hormone manipulation ($P > 0.05$) (Table 3). Overall, more males shed virus in saliva than females 10 days ($\chi^2 = 3.82$, $df = 1$, $P = 0.051$) and 30 days ($\chi^2 = 8.19$, $df = 1$, $P < 0.05$) after inoculation with Seoul virus (Table 3). The prevalence of Seoul virus in feces also differed between males and females on day 30 postinoculation; more males shed virus in feces than females

TABLE 2. Plasma anti-Seoul virus IgG responses[a]

Group	Anti-Seoul virus IgG response (mean ± SE) on day postinoculation[b]					
	0	10	15	20	30	40
Intact males	0.8 ± 0.6	4.9 ± 3.0	84.0 ± 22.0	106.0 ± 19.0*	332.0 ± 47.0*	342.1 ± 56.0*
Castrated males	1.0 ± 0.7	1.0 ± 1.0	82.0 ± 21.0	106.0 ± 27.0*	280.0 ± 71.0*	387.3 ± 84.0*
T-treated males	1.0 ± 0.7	2.0 ± 0.9	33.0 ± 10.0	108.0 ± 14.0*	314.0 ± 41.0*	426.7 ± 43.0*
Intact females	3.0 ± 1.0	9.0 ± 4.0	36.0 ± 10.0	60.0 ± 14.0	189.0 ± 55.0	219.6 ± 63.0
Ovx females	2.0 ± 0.8	4.0 ± 2.0	7.0 ± 3.0	54.0 ± 16.0	187.0 ± 56.0	209.2 ± 53.0
E_2-treated females	3.0 ± 1.0	8.0 ± 2.0	19.0 ± 6.0	39.0 ± 8.0	178.0 ± 42.0	209.1 ± 39.0

[a] Plasma anti-Seoul virus IgG responses in males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] Data are presented as IgG units, in which the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. An asterisk indicates that males had higher IgG responses than females, regardless of hormone manipulation, based on an analysis of variance ($P < 0.05$).

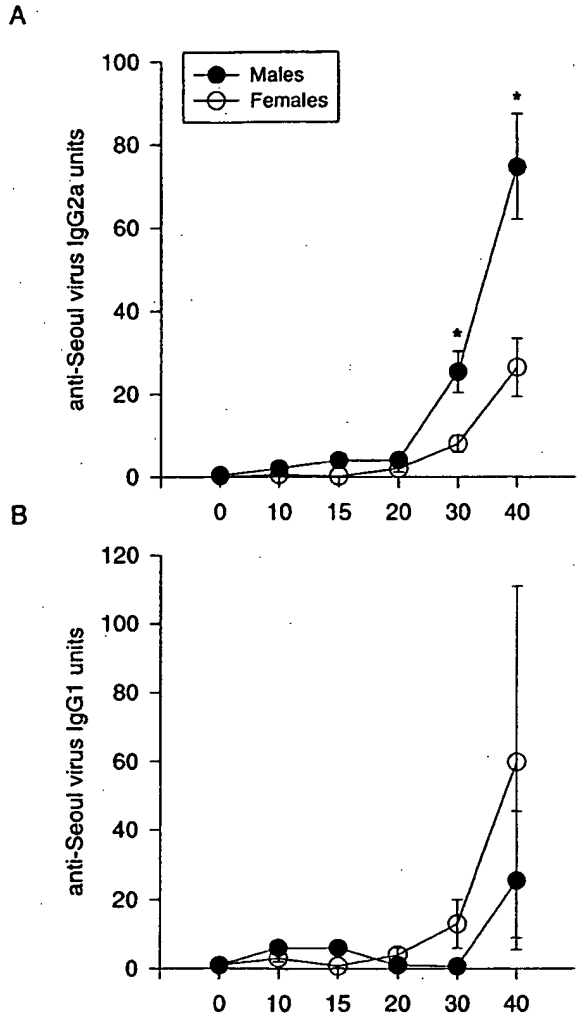


FIG. 2. (A) Plasma anti-Seoul virus IgG2a responses (mean ± standard error) in male and female rats. (B) Plasma anti-Seoul virus IgG1 responses (mean ± standard error) in male and female rats. Blood samples were collected 0, 10, 15, 20, 30, and 40 days following inoculation with Seoul virus. For calculation of IgG2a or IgG1 units, the mean OD of each test sample was divided by the OD of the positive control sample run on the same microtiter plate. Because neither gonadectomy nor hormone replacement had an effect on antibody production, responses from the different treatments groups were collapsed and graphed together. An asterisk indicates that males had higher IgG2a responses than females ($P < 0.05$).

($\chi^2 = 6.88, df = 1, P < 0.05$) (Table 3). In general, males shed virus in saliva and feces more consistently than females, regardless of hormone manipulation (Table 3). The PCR product obtained from saliva and feces of males and females was sequenced and verified as Seoul virus DNA.

Sex differences in the prevalence of hantavirus infection have been observed in several natural rodent populations, including deer mice, brush mice, harvest mice, bank voles, and cotton rats (4, 8, 11, 19, 20, 27). In each case, males are infected more often than females. Field studies of Norway rats suggest that sex differences in hantavirus prevalence reflect sex differences in behaviors, like aggression, that increase the likelihood of males being infected (10). High circulating testosterone concentrations increase the probability of engaging in aggressive encounters in several vertebrate species (21). In addition to modulating aggression, sex steroid hormones can affect immune responses against infection. Studies of viral infections, such as coxsackievirus, suggest that sex differences in both the prevalence and intensity of infection are due to differences in endocrine-immune interactions (12, 13).

Despite the known effects of sex steroids on infection, in the present study, manipulation of adult sex steroids had no effect on immune responses or virus shedding following exposure to Seoul virus. Specifically, males had higher antibody responses and shed virus longer than females, regardless of adult hormone manipulation. Sex steroid hormones affect physiology and behavior at two distinct times during ontogeny (2, 16, 23). During perinatal development, sex steroids cause sex differences in the differentiation or organization of central and peripheral structures. In adulthood, exposure to sex steroids serves to activate preexisting hormonal circuits. The data from the present study may suggest that sex steroid hormones are not involved in hantavirus infection. Alternatively, these data may illustrate that manipulation of activational sex steroids does not alter responses to infection because the hormonal circuitry was organized earlier during development. If sex steroids organize adult responses to infection, then manipulation of neonatal sex steroids should alter adult responses to hantavirus infection.

Regardless of hormone manipulation, males had higher anti-Seoul virus IgG2a responses than females. Recent data from our laboratory indicate that following Seoul virus inoculation, males have elevated IL-2 and gamma interferon concentrations and females have elevated IL-4 responses (S. L. Klein and G. E. Glass, unpublished data). Taken together, these data suggest that males may have higher Th1 responses to hantavirus infection than females. Studies of other viral infections in rodents suggest that females typically have higher Th2 re-

TABLE 3. Virus shedding[a]

Sample and group	No. of virus-shedding rats/total on day postinoculation[b]				
	10	15	20	30	40
Saliva samples					
Intact males	6/11	7/10	6/11	6/11	6/11
Castrated males	4/9	4/9	6/9	5/9	8/9
T-treated males	9/10	7/10	4/10	6/10	7/10
Total males	19/30*	18/29	16/30	17/30*	21/30
Intact females	3/9	6/9	5/9	2/9	2/9
Ovx females	4/10	7/10	2/10	2/10	6/10
E_2-treated females	3/10	10/10	3/10	1/10	6/10
Total females	10/29	23/29	11/29	5/29	14/29
Fecal samples					
Intact males	5/11	4/11	4/11	5/11	1/11
Castrated males	6/9	5/9	7/9	4/8	1/9
T-treated males	4/10	6/10	7/10	7/9	1/10
Total males	15/30	15/30	18/30	16/29*	3/30
Intact females	7/9	4/9	4/9	1/8	0/9
Ovx females	9/10	4/10	6/10	2/10	2/10
E_2-treated females	6/9	5/10	8/10	2/10	1/10
Total females	22/28	13/29	18/29	5/28	3/29

[a] Virus shedding in saliva and feces from males and females that either were intact, gonadectomized (i.e., males were castrated and females were ovariectomized [Ovx]), or gonadectomized with sex steroids replaced (i.e., gonadectomized males received testosterone [T]-filled capsules and gonadectomized females received estradiol [E_2]-filled capsules).

[b] An asterisk indicates that more males shed virus than females on the respective day postinoculation, based on chi-square analyses ($P < 0.05$).

sponses than males and that this is due, in part, to the effects of estrogens on cytokine production (12). In the present study, females tended to produce higher IgG1 responses than males. In contrast to estrogens, androgens promote differentiation of $CD4^+$ T cells to a Th1 phenotype (12). In the present study, however, castrated and intact males had similar IgG2a responses, suggesting that increased Th1 responses are not contingent on the direct effects of androgens.

High antibody responses in males may indicate that males have more efficient immune responses against infection than females. This outcome seems unlikely given the rapid increase and long duration of virus shedding in males compared to females. Alternatively, males may have higher antibody responses than females because virus replication is increased in males. Higher Th1 responses are associated with increased susceptibility to infections caused by coxsackievirus and Sindbis virus in mice (12, 28). Although quantitative analyses were not conducted, males shed Seoul virus longer than females, suggesting that higher Th1 responses among males may be a consequence of increased virus replication.

In summary, although males and females are equally susceptible to infection with Seoul virus, males shed virus longer and produce higher Th1 responses against Seoul virus than females. Increased virus shedding among males may explain why males are more likely to acquire Seoul virus infection following aggressive encounters among natural populations of Norway rats (10). In the present study, manipulation of adult sex steroid hormones did not alter immune responses or virus shedding following inoculation with Seoul virus. Although sex steroid hormones may not mediate sex differences in response to hantavirus infection, sex differences in infection among adults may be altered by sex steroids earlier during development. Alternatively, sex differences in infection may reflect other neuroendocrine changes, such as differences in glucocorticoids, that may affect responses to Seoul virus infection.

This research was supported by NASA grant NCC5-305 (G.E.G.) and NIH NRSA AI 10324 (S.L.K.).

We thank Connie Schmaljohn and Cindy Rossi for providing hantavirus reagents, Alan Scott and Aimee Marson for assistance with PCR development, and Randy Nelson and Deborah Drazen for assistance with radioimmunoassays. We also thank Diane Griffin and Alan Scott for helpful comments on early drafts of the manuscript.

REFERENCES

1. **Alexander, J., and W. H. Stimson.** 1988. Sex hormones and the course of parasitic infection. Parasitol. Today 4:189–193.
2. **Arnold, A. P., and S. M. Breedlove.** 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19:469–498.
3. **Arthur, R. R., R. S. Lofts, J. Gomez, G. E. Glass, J. W. LeDuc, and J. E. Childs.** 1992. Grouping of hantaviruses by small (S) genome segment polymerase chain reaction and amplification of viral RNA from wild-caught rats. Am. J. Trop. Med. Hyg. 47:210–224.
4. **Bernshtein, A. D., N. S. Apekina, T. V. Mikhailova, Y. A. Myasnikov, L. A. Khlyap, Y. S. Korotkov, and I. N. Gavrilovskaya.** 1999. Dynamics of Puumala hantavirus infection in naturally infected bank voles (*Clethrionomys glareolus*). Arch. Virol. 144:2415–2428.
5. **Bijlsma, J. W. J., M. Cutolo, A. T. Masi, and I. C. Chikanza.** 1999. The neuroendocrine immune basis of rheumatic diseases. Trends Immunol. 20: 298–301.
6. **Bodner, L., and B. J. Baum.** 1985. Characteristics of stimulated parotid gland secretion in the aging rat. Mech. Ageing Dev. 31:337–342.
7. **Childs, J. E., G. W. Korch, G. E. Glass, J. W. LeDuc, and K. V. Shah.** 1987. Epizootiology of hantavirus infections in Baltimore: isolation of a virus from Norway rats, and characteristics of infected rat populations. Am. J. Epidemiol. 126:55–68.
8. **Childs, J. E., T. G. Ksiazek, C. F. Spiropoulou, J. W. Krebs, S. Morzunov, G. O. Maupin, K. L. Gage, P. E. Rollin, J. Sarisky, R. E. Enscore, J. K. Frey, C. J. Peters, and S. T. Nichol.** 1994. Serologic and genetic identification of *Peromyscus maniculatus* as the primary reservoir for a new hantavirus in the southwest United States. J. Infect. Dis. 169:1271–1280.
9. **de Savigny, D., and A. Voller.** 1980. The communication of ELISA data from laboratory to clinician. J. Immunoass. 1:105–128.
10. **Glass, G. E., J. E. Childs, G. W. Korch, and J. W. LeDuc.** 1988. Association of intraspecific wounding with hantaviral infection in wild rats (*Rattus norvegicus*). Epidemiol. Infect. 101:459–472.
11. **Glass, G. E., W. Livingstone, J. N. Mills, W. G. Hlady, J. B. Fine, W. Biggler, T. Coke, D. Frazier, S. Atherley, P. E. Rollin, T. G. Ksiazek, C. J. Peters, and J. E. Childs.** 1998. Black Creek Canal virus infection in *Sigmodon hispidus* in southern Florida. Am. J. Trop. Med. Hyg. 59:699–703.
12. **Huber, S. A., J. Kupperman, and M. K. Newell.** 1999. Hormonal regulation of $CD4^+$ T-cell responses in coxsackievirus B3-induced myocarditis in mice. J. Virol. 73:4689–4695.
13. **Huber, S. A., and B. Pfaeffle.** 1994. Differential Th_1 and Th_2 cell responses in male and female BALB/c mice infected with coxsackievirus group B type 3. J. Virol. 68:5126–5132.
14. **Hutchinson, K. L., P. E. Rollin, W.-J. Shieh, S. Zaki, P. W. Greer, and C. J. Peters.** 2000. Transmission of Black Creek Canal virus between cotton rats. J. Med. Virol. 60:70–76.
15. **Klein, S. L.** The effects of hormones on sex differences in infection: from genes to behavior. Neurosci. Biobehav. Rev., in press.
16. **Konstadoulakis, M. M., K. N. Syrigos, C. N. Baxevanis, E. I. Syrigou, M. Papamichail, P. Peveretos, M. Anapliotou, and B. C. Golematis.** 1995. Effect of testosterone administration, pre- and postnatally, on the immune system of rats. Horm. Metab. Res. 27:275–278.
17. **Mankau, S. K., and R. Hamilton.** 1972. The effect of sex and sex hormones on the infection of rats by *Trichinella spiralis*. Can. J. Zool. 50:597–602.
18. **Mertz, G. J., B. L. Hjelle, and R. T. Bryan.** 1997. Hantavirus infection. Adv. Intern. Med. 42:369–421.
19. **Mills, J. N., J. M. Johnson, T. G. Ksiazek, B. A. Ellis, P. E. Rollin, T. L. Yates, M. O. Mann, M. R. Johnson, M. L. Campbell, J. Miyashiro, M. Patrick, M. Zyzak, D. Lavender, M. G. Novak, K. Schmidt, C. J. Peters, and J. E. Childs.** 1998. A survey of hantavirus antibody in small-mammal populations in selected United States national parks. Am. J. Trop. Med. Hyg. 58:525–532.
20. **Mills, J. N., T. G. Ksiazek, B. A. Ellis, P. E. Rollin, S. T. Nichol, T. L. Yates, W. L. Gannon, C. E. Levy, D. M. Engelthaler, T. Davis, D. T. Tanda, J. W. Frampton, C. R. Nichols, C. J. Peters, and J. E. Childs.** 1997. Patterns of association with host and habitat: antibody reactive with Sin Nombre virus in small mammals in the major biotic communities of the southwestern United States. Am. J. Trop. Med. Hyg. 56:273–284.
21. **Nelson, R. J.** 2000. An introduction to behavioral endocrinology, 2nd ed. Sinauer Associates, Inc., Sunderland, Mass.
22. **Nuzum, E. O., C. A. Rossi, E. H. Stephenson, and J. W. LeDuc.** 1988. Aerosol transmission of Hantaan and related viruses to laboratory rats. Am. J. Trop. Med. Hyg. 38:636–640.
23. **Pheonix, C. H., R. W. Goy, A. A. Gerall, and W. C. Young.** 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65:369–382.
24. **Rajan, T. V., F. K. Nelson, L. D. Shultz, K. L. Shultz, W. G. Beamer, J. Yates, and D. L. Greiner.** 1994. Influence of gonadal steroids on susceptibility to *Brugia malayi* in scid mice. Acta Trop. 56:307–314.
25. **Smith, E. R., D. A. Damassa, and J. M. Davidson.** 1977. Hormone administration: peripheral and intracranial implants, p. 259–279. *In* R. D. Myers (ed.), Methods in psychobiology. Academic Press, New York, N.Y.
26. **Tiuria, R., Y. Horii, S. Tateyama, K. Tsuchiya, and Y. Nawa.** 1994. The Indian soft-furred rat, *Millardia meltada*, a new host for *Nippostrongylus brasiliensis*, showing androgen-dependent sex difference in intestinal mucosal defence. Int. J. Parasitol. 24:1055–1057.
27. **Weigler, B. J., T. G. Ksiazek, J. G. Vandenbergh, M. Levin, and W. T. Sullivan.** 1996. Serological evidence for zoonotic hantaviruses in North Carolina rodents. J. Wildl. Dis. 32:354–357.
28. **Wesselingh, S. L., B. Levine, R. J. Fox, S. Choi, and D. E. Griffin.** 1994. Intracerebral cytokine mRNA expression during fatal and nonfatal alphavirus encephalitis suggests a predominant type 2 T cell response. J. Immunol. 152:1289–1297.
29. **Zuk, M., and K. A. McKean.** 1996. Sex differences in parasite infections: patterns and processes. Int. J. Parasitol. 26:1009–1024.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☑ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.